Food Research

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Volume 22, Number 4 *July-August, 1957*

AN OFFICIAL PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS

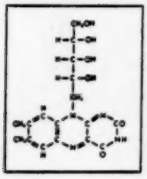
INSIDE SCIENCE

The Vital Story

A Quick History. Independent investigators, working separately to unlock several of nature's doors, sometimes open up unsuspected relationships. This happened with vitamin B_2.

Investigations. About 25 years ago, several groups, notably Warburg's, were investigating a "yellow enzyme" obtained from yeast. Almost simultaneously other investigators were studying a food factor that aided growth of laboratory animals.

What they found. Proceeding with chemical analysis of this growth factor, the team of Kuhn, György, and Wagner-Jauregg noted a relationship between the growth-producing agent and the "yellow enzyme." Their findings, and those of other researchers along similar lines, were published in 1933. Eventually, riboflavin and an essential part of the yellow enzyme were found to be identical and the unity of an essential nutrient and cellular metabolism was established.



Isolation of pure riboflavin was achieved by Kuhn and his co-workers, and by Ellinger and Koschara, in 1933.

Nomenclature. Known in the United States as riboflavin, this vitamin has also been called lactoflavin, ovoflavin, hepatoflavin, and vitamin G.

SYNTHESIS

By 1935, two eminent chemists, working separately, had synthesized riboflavin, practically in a dead heat. Prof. Paul Karrer of the University of Zurich, a collaborator of the Hoffmann-La Roche Laboratories, produced the first successful synthesis. Five weeks later Richard Kuhn of Germany announced his synthesis of the vitamin. Prof. Karrer subsequently shared the Nobel Prize in Chemistry for his work in vitamins and carotenoids.

The Karrer synthesis forms the basis for chemical processes in widespread use today by Hoffmann-La Roche and other leading manufacturers throughout the world. Riboflavin is also manufactured today by fermentation methods.

CHEMICAL AND PHYSICAL PROPERTIES

Riboflavin is yellow, slightly water-soluble with a greenish fluorescence and a bitter taste. Its empirical formula is $C_{17}H_{20}N_4O_6$. Vitamin B_2 produced by the Roche process is identical in every way with that occurring in nature.

How does vitamin B_2 work? Riboflavin is a vital part of nature's chain of reactions for utilization of carbohydrate energy. It has been found to be a constituent of many enzyme systems and is thus intimately connected with life processes. It is probably required by the metabolic processes of every animal and bird as well as by many fishes, insects and lower forms of life. (In certain animals, however, the requirement may be synthesized by bacteria within the intestine.)

In the cells riboflavin goes to work attached to a phosphate group. This substance, known as riboflavin-5'-phosphate or flavin mononucleotide, may in turn be attached to still another essential substance, adenylic acid, forming flavin adenine dinucleotide. Either nucleotide then is attached to protein, thereby forming an enzyme, and takes its part in oxidation-reduction reactions.

Requirements in Human Nutrition. As we have seen, vitamin B_2 is essential to life. We have no special storage organs in our bodies for this vitamin, although a certain level is maintained in various tissues, with relatively large amounts found in the liver and kidneys.

MEASURING METHODS

In the beginning, riboflavin activity was described in "Bourquin-Sherman units" and requirements were thought to be very small. Subsequent research showed otherwise. Milligrams of weight became the unit and the Food & Drug Administration of the U. S. Dept. of Health, Education & Welfare has established a minimum daily requirement of 2.0 mg. of riboflavin for all persons 12 or more years old. For infants it is 0.5 mg. These requirements are designed to prevent the occurrence of symptoms of riboflavin deficiency disease. The minimum daily requirement for this vitamin for children from 1 to 12 years has not been established by the F. & D. A.

Recommended allowances. The Food & Nutrition Board of the National Research Council has recommended the following daily dietary allowances of riboflavin, expressed as milligrams. These are designed to maintain good nutrition of healthy persons in the U. S. A.

			Boys	Girls
Men			1.6	
Women			1.4	
"	(3rd trimester of pregnancy)		2.0	
"	(Lactating)		2.3	
Infants,	1-3	months	0.4	
"	4-9	"	0.7	
"	10-12	"	0.9	
Children,	1-3	years	1.0	
"	4-6	"	1.2	
"	7-9	"	1.5	
Adolescents,	10-12	years	1.8	1.6
"	13-15	"	2.1	2.0
"	16-20	"	2.3	1.9

# of VITAMIN B_2 *by* Science Writer

(Riboflavin)

Deficiencies of vitamin B_2 appear in several ways in human beings. The eyes, the skin, the nerves, and the blood show the effects of too little riboflavin. Laboratory animals have demonstrated that a riboflavin-deficient diet can cause death of adults and can slow or stop growth in the young. Female animals, deprived of riboflavin in the diet, may produce offspring with congenital malformations.

Medical uses. To overcome and control deficiencies in human beings, physicians have pure riboflavin available for administration by injection or orally, by itself or with other "B" vitamins or multi-vitamin-mineral combinations.

How do we get our daily riboflavin? Vitamin B_2 has wide distribution throughout the entire animal and vegetable kingdoms. Good sources are milk and its products, eggs, meats, legumes, green leaves and buds. Whole-grain cereals have significant but not large amounts of riboflavin.

ADDITION TO FOODS

Cereal foods play a large part in our diet. To produce the white flour almost all of us want, millers are obliged to remove parts of the wheat that contain much of the grain's riboflavin and other nutrients. In addition, cereal grains are not rich sources of riboflavin. Millers meet this problem by *enriching* the grain foods for which federal standards exist with vitamins B_1, B_2, niacin and the mineral iron. In the case of vitamin B_2, however, they do more than *restore* the processed food to its natural riboflavin level; they *fortify* the food with enough of this essential vitamin to make it nutritionally more valuable than it was in nature.

Acting to protect the good health of millions of Americans, bakers and millers adopted *enrichment* of white bread and white flour in 1941. Since that time, other foods, such as macaroni products, corn meal and grits, farina, pastina and breakfast cereals have had their food value increased by enrichment with pure riboflavin and other vitamins and minerals.

When enriching, fortifying or restoring, food manufacturers add the necessary quantity of riboflavin (and other vitamins and minerals) to the food during processing, so that the finished product meets federal, state, and territorial requirements or contributes to the consumer an amount of the vitamin that dietary experts believe significantly useful.

PRODUCTION

Prof. Karrer's synthesis of riboflavin was a laboratory success. Adapting the process to commercial production, however, demanded original thinking by chemists at Hoffmann-La Roche. The production of riboflavin by chemical synthesis requires the production of ribose, a rare sugar, at an early stage in the process. This special sugar must be made inexpensively if the synthesis is to be practical. Sugar chemistry is a difficult matter. In a brilliant piece of work, the Roche chemical experts developed a method to produce ribose on a commercial scale by an electrolytic process, thus overcoming a most troublesome problem. Subsequently, Roche chemists developed the first practical synthesis for riboflavin-5'-phosphate, identical with natural flavin mononucleotide.

Picture three streams joining to form a river and you have a simplified idea of the Roche process for synthesizing vitamin B_2. O-xylene and glucose are processed separately to form xylidine and ribose respectively. These are joined to form ribitylxylidine, which is then converted to ribitylaminoxylidine. Starting separately with malonic ester, which is processed through intermediate stages to alloxan, the third "stream" is then joined with ribitylaminoxylidine to form riboflavin. Purification occurs at each step of the synthesis. Riboflavin 'Roche' equals or exceeds U. S. P. standards.

By the tons. So efficient is the Roche process that pure riboflavin is produced *by the tons* for use in pharmaceutical products and processed foods. An interesting development by Roche is the production of riboflavin in different forms related to the method of end use. 'Roche' Regular riboflavin U. S. P. is especially useful in dry enrichment premixes, powdered dietary supplements, pharmaceutical tablets and soft gelatin capsules. 'Roche' Solutions type is preferred for the manufacture of solutions having low concentration. 'Roche' Riboflavin-5'-Phosphate Sodium is a highly and rapidly soluble riboflavin compound favored for all pharmaceutical liquid products and some tablets, lozenges, and capsules. It has a more pleasant taste than the bitter U. S. P. riboflavin.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better using pure riboflavin 'Roche.' Reprints of this and others in the series will be supplied on request without charge. Also available without cost is a brochure describing the enrichment or fortification of cereal grain products with essential vitamins and minerals. These articles and the brochure have been found most helpful as sources of accurate information in brief form. Teachers especially find them useful in education. Regardless of your occupation, feel free to write for them. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul St., West; Montreal, Que.

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

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FOOD TECHNOLOGY . . .	$8.50 Domestic; $10.00 Canada, Cuba, and Mexico; $12.00 all other foreign.

Send Subscriptions to

FOOD TECHNOLOGY

THE GARRARD PRESS

510-522 N. Hickory Street

CHAMPAIGN, ILLINOIS

Food Research

An Official Publication of

The Institute of Food Technologists

Volume 22 JULY–AUGUST, 1957 Number 4

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Printed in U. S. A.

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-522 N. Hickory Street, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

Volume 22 JULY–AUGUST, 1957 Number 4

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SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $7.50 per volume.

Non-members: Domestic, $8.50 per volume; foreign, $10.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.50; foreign, $1.75.

CORRESPONDENCE

Editorial: MARTIN S. PETERSON, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD RESEARCH, 510-522 N. Hickory Street, Champaign, Ill.

Member Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

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LINDANE RESIDUE IN CUCUMBER FERMENTATION[a, b, c]

MELVIN R. JOHNSTON
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(Manuscript received June 13, 1956)

Within recent years many synthetic pesticides have been developed and used for the protection of food crops. These powerful pest control agents have received much attention from investigators both as to their toxicological nature and their ability to alter food flavor.

The influence of pesticide residues on food fermentations has not been so widely studied as the flavor effect. The problem has, however, received the attention of Adams *(1)* who, working with the fungicide captan, found that yeast fermentation of cherry juice was delayed 24 hours and 30 days by 7 and 10 p.p.m., respectively, of the material.

Lindane, the gamma isomer of hexachlorocyclohexane, is a very effective insecticide in controlling certain cucumber insects. A metabolic relationship between lindane and i-inositol has been reported by several investigators for yeasts *(11, 13, 15)*, mold *(3, 9)*, other organisms *(4, 10)* and enzymes *(7, 12, 14)*. The study reported here was made to ascertain the influence of lindane residue on the cucumber fermentation and on certain species of the microflora.

EXPERIMENTAL

Materials and methods. To study the effect of lindane spray residue on cucumber fermentation, the lindane was introduced by 3 methods: spraying harvested cucumbers just prior to brining; using recommended insect control schedules with lindane on replicated experimental field plots; and adding known amounts of pure lindane and i-inositol to highly defined liquid media for growing pure cultures of micro-organisms.

For the first method (1953), harvested cucumbers were divided into two approximately equal lots. One lot was used for the controls. The other lot was sprayed with lindane (1 lb. 25% wettable per 100 gal.) as follows: cucumbers were spread one layer thick on a tarpaulin and the spray was applied uniformly over the sample by single passes of the nozzle held about 18 inches above the cucumbers. The control lot was sprayed with tap water only. Three replications of each treatment were made. One hundred pounds of cucumbers were weighed into each 21-gallon keg and covered with 10 gallons of brine. The concentration of the brine for the first replication was 45° Salometer; this brine density was maintained by addition of salt during the active fermentation period. Brine concentration for the second replication was also 45°, but in this case it was permitted to equalize with the cucumbers. Cucumbers in the third replication were covered with a 35° Salometer brine which was permitted to equalize with the cucumbers during the fermentation period. The growth of film yeasts during the active fermentation was suppressed by a G-E Sun lamp and the ambient air temperature of the room was not controlled.

Cucumbers from replicated insect-disease control field plots were used for the second method (1954). Four treatment schedules and a control were employed; namely, Block I

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[a] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, Missouri, June 12, 1956.

[b] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 1635.

[c] Based on a dissertation submitted, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, to the Faculty of the University of Missouri.

replications 1 and 6: 1% lindane dust and a 7.5% captan dust; Block II replications 2 and 7: lindane spray (1 lb. 25% wettable per 100 gal.); Block III replications 3 and 8: combination of lindane (1 lb. 25% wettable per 100 gal.) plus captan (2 lb. 50% wettable per 100 gal.); Block IV replications 4 and 9: combination spray of lindane (1 lb. 25% wettable per 100 gal.) and methoxychlor (2 lb. 50% wettable per 100 gal.); and Block V replications 5 and 10; control (not treated). Plots were treated 7 times prior to harvesting the experimental sample, with the last application the previous day. One hundred pounds of field run cucumbers (25 lbs. from each field replication) were weighed into each 21-gallon keg and covered with 10 gallons of 40-degree Salometer brine. The brine was permitted to equalize with the cucumbers during the fermentation period. Film formers were suppressed by a G-E Sun lamp and the ambient air temperature of the room was not controlled.

The course of fermentation in each replication was followed by titration of the developed acidity and by bacteriological examinations of the cucumber brines. Acid production was determined by a Fisher Electric Titrimeter with a 7.5 pH endpoint using 10 ml. of fermenting brine with 40 ml. of double distilled water which was brought to a boil prior to titration.

Differential media for the bacteriological examination of the brine were: V-8 medium developed by Fabian *et al. (6)* for the differentiation of *lactobacilli* and an acidified (pH 3.5) tryptone-glucose-beef extract medium by Difco *(5)* for yeasts. Brine samples for analysis were siphoned from the kegs with a large aliquot (100 ml.) taken first for chemical analysis and then a small aliquot (10 ml.) placed in a sterile cotton plugged test tube for bacteriological analysis.

Petri dishes were poured from index dilutions of the brine and the number of dilutions necessary to facilitate counting the plates was estimated. At least two dilution levels were used on a sample and after the agar had solidified, the petri dishes were inverted and incubated. For the V-8 and acidified dextrose media the incubation was at 20° C. for 48 and 72 hours, respectively. The remainder of the bacteriological sample (about 8 ml.) from each replication was used for the turbidity measurement on an Evelyn colorimeter with a 650 mμ filter. The instrument was standardized at 100% transmittance with distilled water.

Pure cultures of yeasts, bacteria and mold were exposed to lindane and i-inositol in highly defined liquid media. The yeasts were obtained from the Northern Utilization Research Branch, U. S. Department of Agriculture, Peoria, Illinois. NRRL numbers and names are Y-301 *(Candida krusei)*, Y-659 *(Schizosaccharomyces pombe)*, Y-862 (Bakers yeast), Y-1011 *(Zygosaccharomyces acidifacians)*, Y-1089 *(Saccharomyces carlsbergensis)*, Y-1567 *(Torulaspora rosei)*, Y-2007 *(S. fragilis)*, Y-2021 *(Debaromyces globosus)*, and Y-2240 *(Hansenula fabianii)*. Inoculum cultures were grown on acidified dextrose nutrient agar slants. Culture slants were incubated at room temperature for 24 hours prior to being used for inoculating the liquid medium.

The inositol assay medium of Wooley *(17)* and certain techniques of Williams *et al. (16)* were used. A large volume of the assay medium was made and stored at room temperature. A stock solution (100 p.p.m.) of sterile inositol was made. The lindane stock solution (10,000 p.p.m.) was prepared in 95% ethyl alcohol. Just prior to making a test, a stock suspension (400 p.p.m.) of lindane was made by mixing one milliliter of the lindane in alcoholic solution with 24 ml. of sterile distilled water. Initially it was a milky suspension and after about 30 minutes it would coagulate into large curds. It was used immediately after preparation and the remainder was discarded.

A standard technique was developed for studying the effect of lindane and inositol on the cell proliferation of the previously named yeasts. An inoculum was prepared by washing four loops of cells in two 40 ml. volumes of sterile distilled water. The cells were removed from the wash water by centrifugation at 2400 r.p.m. for 2 minutes. They were resuspended in a third 40 ml. volume of sterile distilled water which was the inoculum. Ten milliliters of the basal medium and 10 ml. sterile distilled water were transferred to each of 8 sterile cotton plugged 125 ml. Erlenmeyer flasks. One milliliter of inoculum was added to each flask. The amount of lindane and i-inositol added to paired flasks was: first pair—1 ml. sterile distilled water and 1 ml. lindane suspension; second pair—1 ml. inositol solution and 1 ml. lindane suspension; third pair—1 ml. inositol solution and 1 ml. sterile distilled water; fourth pair—2 ml. sterile distilled water. The incubation period at room temperature was dependent on the growth rate of each species.

A turbidity measurement with a "Spectronic 20" at 625 mμ was used to indicate the relative amount of cell production in the four treatments.

Bacterial species, 23-2 *(Lactobacillus plantarum)* and G-4 *(Aerobacter aerogenes)*, were obtained from the collection of the Dairy Husbsandry department. The technique used to check the effect of lindane and inositol on the growth of *A. aerogenes* was the same as the yeast technique (medium and stock solutions). To facilitate the growth of *L. plantarum*, the yeast medium was fortified with 3 ml. of a 50-50 V-8 vegetable juice-water dilution. With this exception the technique and materials were the same as for the yeast work.

An inositolless mutant strain of *Neurospora crassa* (A-3507) was obtained from the Northern Utilization Research Branch at Peoria. The influence of lindane and inositol on the growth of this mold was determined in a manner similar to that described for the yeast. The basal medium and bioassay technique developed by Beadle *(2)* was used for growing and harvesting the mycelium. The same stock solutions of lindane and inositol were used as described for the yeast.

TABLE 1

Effect of lindane on lactic acid production in cucumber brines, 1953

Date	Milliliters of N/10 NaOH per 10 ml. of brine					
	1	2	3	4	5	6
7-19	.6	.6	1.7	1.4	2.3	2.1
7-21	.5	.5	3.8	4.0	4.7	3.7
7-22	.5	.6	4.8	4.8	5.4	4.5
7-23	.7	.7	6.0	6.0	6.8	5.7
7-24	.7	.7	5.6	5.3	7.0	6.1
7-25	.7	.6	5.9	5.3	7.0	6.4
7-27	.8	.7	6.3	5.8	7.2	7.0
7-28	1.2	1.3	6.2	5.7	7.1	7.1
7-29	1.4	.8	6.4	5.3	6.9	7.1
7-30	.9	.9	6.5	5.4	7.1	7.5
7-31	1.7	1.1	6.3	5.9	7.3	7.1
8- 1	1.0	1.1	6.6	5.8	7.5	7.7
8- 3	1.1	1.4	6.4	6.1	6.5	7.2
8- 4	1.3	1.2	7.1	6.0	6.9	7.6
8- 5	1.3	1.6	6.9	6.3	6.9	7.6
8- 7	1.5	1.6	5.6	5.6	6.4	6.9
8- 8	1.3	1.5	5.9	5.2	5.9	6.5
8-10	1.2	1.2	5.6	5.1	5.5	6.3
8-12	1.3	1.3	5.4	4.9	6.1	6.3
8-14	1.2	2.0	5.6	4.9	4.6	7.0
Total	20.9	21.4	114.6	104.8	125.1	127.4
Mean	1.05	1.07	5.73	5.24	6.26	6.37

Analysis of variance of acid production

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	119		
Treatment	1	0.41	3.67 *
Salting	2	319.34	3,091.32 **
Dates	19	4.37	38.65 **
Treat. x Salting	2	1.07	7.08 **
Treat. x Dates	19	0.14	0.94
Salting x Dates	38	0.829	5.42 **
Treat. x Dates x Salting	38	0.15	

L.S.D. .05 p = 0.25
.01 p = 0.33

TABLE 2

Effect of lindane on turbidity of cucumber brines, 1953

Date	% transmittance of brines at 650 mμ					
	1	2	3	4	5	6
7-17	99.0	99.0	98.0	98.5	83.0	89.0
7-18	99.0	98.0	95.0	98.5	77.0	81.5
7-19	99.5	99.0	70.0	76.5	57.0	57.5
7-20	96.5	97.0	58.0	68.5	57.0	62.0
7-21	94.0	94.0	46.0	65.0	47.0	64.0
7-22	95.5	97.0	42.0	76.0	49.0	65.0
7-23	96.0	97.5	44.0	80.0	53.0	61.0
7-24	96.0	96.5	45.5	71.5	46.0	50.0
7-25	97.0	98.0	55.0	78.0	56.0	57.0
7-27	97.5	97.5	73.0	85.0	71.0	70.0
7-28	97.5	98.0	82.0	89.0	79.0	79.0
7-29	97.5	95.5	87.0	91.0	83.0	85.0
7-30	96.1	95.5	88.0	89.5	83.0	86.0
7-31	97.5	96.5	76.0	80.0	75.0	76.0
8- 1	98.0	96.5	80.0	82.0	79.5	77.5
8- 3	97.5	96.5	87.0	90.0	86.5	86.0
8- 4	98.5	99.0	87.0	91.5	87.0	86.5
8- 5	97.0	98.0	89.5	93.5	88.0	88.5
8- 7	92.5	89.0	73.0	74.0	77.0	72.0
8- 8	88.5	90.5	79.0	78.0	81.0	83.0
8-10	96.0	94.0	81.0	88.0	86.5	80.0
8-12	87.5	93.5	84.0	89.0	82.5	86.0
8-14	95.5	93.5	72.5	73.5	76.5	74.0
Total	2,209.6	2,209.5	1,692.5	1,906.5	1,660.5	1,716.5
Mean	96.06	96.06	73.58	83.89	72.19	74.63

Analysis of variance of brine turbidity

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	137		
Treatment	1	641.82	32.40 **
Salting	2	6,550.07	330.69 **
Dates	22	421.12	21.26 **
Treat. x Salting	2	210.96	10.65 **
Treat. x Dates	22	35.47	1.79
Salt x Dates	44	120.01	6.06 **
Treat. x Salt x Dates	44	19.81	

L.S.D. .05 p = 2.64
.01 p = 3.53

RESULTS AND DISCUSSION

Production of acid in the fermentations as measured by titrable acidity was found to be stimulated by the presence of lindane residue. As the data in Tables 1 and 5 indicate, the magnitude of the stimulation was not very great and unaccountable variability within each year's experiment was apparent. The effect of treatment on acid production was statistically significant at the 5% level for the 1953 experiment and at the 1% level for the 1954 experiment. A lack of higher significance in the 1953 experiment might be explained by the masking effect which brine density of the replicated treatments had on acid production. The effect of brine density on acid production

TABLE 3

Effect of lindane on lactic acid bacteria in cucumber brines, 1953

Date	Cell counts in 10,000 per ml. of brine on V-8 agar					
	1	2	3	4	5	6
7-18	.8	1.3	63.0	142.0	460.0	630.0
7-19	30.0	38.0	1,890.0	2,730.0	4,940.0	5,580.0
7-20	11.0	78.0	790.0	630.0	1,080.0	6,500.0
7-21	24.0	81.0	19,900.0	10,500.0	11,700.0	2,700.0
7-22	34.0	90.0	19,000.0	5,000.0	6,400.0	2,800.0
7-23	34.0	90.0	15,000.0	3,000.0	5,700.0	8,000.0
7-24	10.0	10.0	9,600.0	3,600.0	6,300.0	7,600.0
7-25	10.0	10.0	7,900.0	2,300.0	3,400.0	4,600.0
7-27	5.0	10.0	2,600.0	370.0	550.0	620.0
7-30	40.0	20.0	50.0	110.0	30.0	80.0
8- 1	14.0	11.0	14.0	16.0	30.0	20.0
8- 3	15.0	11.0	.9	1.3	2.9	1.2
8- 5	1.3	8.0	.5	.6	1.7	.4
Total	229.1	458.3	76,808.4	28,399.9	40,594.6	39,131.6
Mean	17.6	35.2	5,908.3	2,184.6	3,122.6	3,010.1

Analysis of variance of lactobacilli

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	77		
Treatment	1	31,598,616	6.19 **
Salting	2	114,239,884	22.37 **
Dates	12	40,135,305	7.86 **
Treat. x Salt	2	29,307,918	5.74 **
Treat. x Dates	12	8,672,614	1.70
Salt. x Dates	24	15,389,095	3.01 **
Salt. x Dates x Treat.	24	5,105,817	

L.S.D. .05 p = 1,828
.01 p = 2,478

was significant at the 1% level under the conditions of this experiment. As shown in Table 1, the 45-degree Salometer brine during the active fermentation strongly inhibited acid production in the 1 and 2 sample. As to the practical significance of the observed differences in the data due to treatment, it might be questionable (Table 1).

Percent transmission at 650 mμ of the fermenting brines was found to be affected by the lindane residue as shown by the data in Tables 2 and 6. Since percent transmission is inversely proportional to the microbial cell density of the brine; it follows that the differences in cell density due to treatment were highly significant. Lindane-sprayed samples had a lower cell density than the control samples in the 1953 experiment. In comparing the form in which lindane was applied in 1954, it does not appear that the dust affected the cell density; whereas the spray does reduce the cell content. Variability within these data was apparent. The salting schedules of the 1953 experiment had a very pronounced effect on the cell density of the various brines. Microbial cell density was inversely proportional to the salt concentration of the brines. A high salt concentration had a low cell density; whereas a low salt concentration had a high cell density. These differences were established

TABLE 4

Effect of lindane on yeasts in cucumber brines, 1953

Date	Cell counts in 10,000 per ml. of brine on AD agar					
	1	2	3	4	5	6
7-17	.1	.1	.2	.3	.5	.4
7-18	1.5	3.6	315.0	205.0	230.0	300.0
7-19	4.7	6.6	30.0	574.0	217.0	544.0
7-20	1.5	2.7	1,190.0	238.0	880.0	1,480.0
7-21	17.0	15.0	62.0	1,070.0	1,820.0	920.0
7-22	6.1	4.7	6,640.0	1,210.0	14.0	97.0
7-23	3.4	5.4	7,400.0	1,180.0	3,580.0	2,400.0
7-24	2.2	5.9	8,280.0	1,930.0	4,450.0	5,120.0
7-25	9.8	13.0	5,390.0	1,310.0	2,450.0	2,250.0
7-27	6.3	5.7	340.0	480.0	1,040.0	1,230.0
7-30	44.0	13.9	39.0	52.0	37.0	58.0
8- 1	11.7	9.7	41.0	12.4	16.1	7.3
8- 3	9.6	4.4	11.8	6.9	15.1	16.2
8- 5	39.0	4.7	.8	1.9	3.7	1.9
Total	156.9	95.4	29,739.8	8,270.5	14,753.4	14,424.8
Mean	11.2	6.8	2,124.2	590.7	1,053	1,030

Analysis of variance of yeast

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	83		
Treatment	1	5,688,493	4.62 **
Salting	2	13,930,216	11.30 **
Dates	13	6,606,419	5.36 **
Treat. x Dates	13	1,291,349	1.05
Treat. x Salt	2	5,389,427	4.37 *
Salt x Dates	26	2,342,103	1.90
Treat. x Salt x Dates	26	1,232,543	

L.S.D. .05 p = 861
.01 p = 1,164

after about the third day of the fermentations; they persisted throughout the active fermentation period (Table 2).

The microbiological flora undoubtedly represents a basis for explaining the observed effect of lindane residue on the cucumber fermentation. The 2 groups of micro-organisms studied by differential media were found to be affected by the residue of the insecticide. Lactic acid producers were found to be reduced by the lindane residue from a statistical treatment of the data; yet an inspection of the data in Table 3 shows that one replicated pair (samples 3 and 4) of the 3 replications was probably responsible for the determined significance. The other replicated pairs were about the same; and actually in one case, the sprayed samples was slightly higher than the control. As shown in Table 4, the yeasts were found to be inhibited by the lindane residue. The sprayed samples (2, 4 and 6) were lower than the controls by a highly significant amount. As in the case of the lactic acid producers, the yeasts were strongly affected by the brining schedule. In all cases the high density brines had low cell counts and the low density brines had high cell counts. Variations within brining replications in the 1953 experiment were not

TABLE 5

Effect of lindane on lactic acid production in cucumber brines, 1954

Date	Milliliters of N/10 NaOH per 10 milliliters of brine									
	1	2	3	4	5	6	7	8	9	10
7-15	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.3	0.2
7-16	0.3	0.4	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.2
7-17	1.1	1.0	0.8	0.9	0.7	0.6	0.8	1.3	1.5	0.2
7-19	2.5	2.7	2.4	3.5	3.5	3.2	2.8	2.8	3.4	2.3
7-20	3.5	3.8	3.6	4.4	4.2	4.3	3.8	3.3	3.9	4.4
7-21	3.6	4.3	4.3	4.5	4.9	4.8	4.4	3.4	4.1	4.2
7-22	4.1	5.0	5.1	5.4	5.6	5.8	4.9	4.1	4.4	5.3
7-23	4.3	5.6	5.7	5.2	6.6	6.3	5.4	4.7	4.9	6.2
7-24	4.3	5.6	5.6	5.8	6.1	5.9	4.9	4.4	4.6	5.8
7-26	4.6	6.0	6.0	5.6	6.2	6.0	5.2	4.5	5.0	5.9
7-29	3.7	5.7	5.7	5.2	5.7	5.7	4.6	4.2	4.7	5.7
7-30	4.0	5.6	5.6	5.1	5.5	5.7	4.4	4.0	4.5	5.7
8- 5	3.9	5.7	5.7	5.4	5.7	5.7	4.4	4.1	4.7	5.3
8-27	3.5	5.2	5.2	4.9	5.4	5.3	3.9	3.6	5.5	5.1
9-18	3.3	5.2	5.2	4.6	5.1	5.1	3.8	3.3	4.2	4.8
Total	46.8	62.0	61.3	61.0	65.6	65.0	53.9	48.2	56.0	61.3
Mean	3.1	4.1	4.1	4.1	4.4	4.3	3.6	3.2	3.7	4.1

Analysis of variance of acid production

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	149		
Treatment	3	7.00	44.50 **
Treat. x non-treat.	1	4.74	30.13 **
Dates	14	35.64	226.54 **
Replications	1	1.06	6.74 *
Remainder	130	0.157	

L.S.D. .05 p = 0.28
.01 p = 0.37

significant; and therefore, the observed differences in the experimental data cannot be attributed to variations within the duplicate samples of the various salting schedules (Table 3 and 4).

Microbiological data from the insect control schedules of 1954 were not conclusive. Treatment and replication differences were found to be highly significant by the statistical treatment of the data in Tables 7 and 8. Even with the variability of the data, certain trends seem to be present. Lindane dust and control treatment tend to be about the same for *lactobacilli* counts; whereas, the yeast counts from the lindane dust treatment are somewhat higher than the control. The lindane spray treatment samples tend to have lower populations of *lactobacilli* and yeasts than the control samples. The lindane-captan combination spray treatment samples tend to have higher populations of yeasts; whereas the lactobacilli cell counts were lower than the controls. The lindane-methoxychlor combination spray treatment samples tend to have higher populations of yeast; whereas the lactobacilli cell counts were lower than the control samples. Variations in the brine density among the treatments of the 1954 experiment were statistically significant at the 5% level with a least significant difference of 0.51-degree Salometer. It

TABLE 6

Effect of lindane on turbidity of cucumber brines, 1954

Date	% transmittance of brines at 650 mμ									
	1	2	3	4	5	6	7	8	9	10
7-15	97.5	90.5	100.0	92.5	97.0	95.0	92.5	93.4	97.5	97.0
7-16	87.8	75.8	81.4	82.5	87.9	81.7	83.0	74.9	85.5	87.8
7-17	55.0	61.0	59.0	60.0	69.0	77.5	57.0	42.7	42.4	87.1
7-19	29.8	33.3	46.5	25.6	41.5	23.8	31.9	40.1	27.0	20.2
7-20	33.2	36.5	38.5	23.9	42.2	21.3	25.9	30.8	22.6	20.3
7-21	36.8	34.4	42.9	32.8	40.0	22.1	24.5	32.7	25.2	23.8
7-22	45.2	44.2	52.0	41.4	48.2	27.5	27.9	26.4	28.8	31.9
7-23	42.6	46.8	45.8	44.4	23.3	19.0	25.3	28.8	29.2	38.1
7-24	54.6	56.4	51.1	35.0	32.1	25.1	27.4	37.3	33.1	34.9
7-26	55.8	56.7	58.1	44.1	38.6	29.7	27.1	45.0	33.9	34.6
7-29	60.6	62.0	54.8	41.0	50.7	41.6	42.2	53.6	45.1	30.9
7-30	61.7	66.4	59.3	48.3	57.4	44.3	48.1	52.3	34.7	34.4
8- 5	68.2	73.2	60.7	59.2	53.7	43.5	48.6	57.6	58.3	48.9
8-27	70.2	72.2	66.5	65.4	72.1	55.4	67.2	67.9	64.4	70.3
Total	799.0	809.4	816.6	696.1	753.7	607.5	628.6	683.5	627.7	660.2
Mean	57.0	57.8	58.3	49.7	53.8	43.4	44.9	48.8	44.8	47.2

Analysis of variance of turbidity

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	139		
Treatment	3	1,284.5	11.60 **
Treat. x non-treat.	1	312.52	2.82 *
Dates	13	3,287.73	29.69 **
Replications	1	3,180.64	28.72 **
Remainder	121	110.72	

L.S.D. .05 p = 7.85
.01 p = 10.38

would seem that this amount of variation could not account for the observed differences in the experiment data.

Data from the experiment on the effect of lindane and inositol on the growth of pure cultures of yeast, bacteria and mold indicate a relationship between these 2 compounds for certain micro-organisms (Table 9). Of the species of organisms studied, the yeast were most susceptible to inhibition of growth by lindane and in every instance the addition of inositol to the media improved cell production. *Saccharomyces fragilis* (Y-2997) was retarded by lindane and it did not require inositol for cell proliferation. *Debaromyces globosus* (Y-2021) did not require inositol for growth and it was only slightly inhibited by lindane. *Hansenula fabianii* (Y-2240) grew as well in the presence of lindane as it did with inositol or the control. *S. carlsbergensis* (Y-1089), a bioassay organism for inositol, was strongly retarded by lindane. *Schizosaccharomyces pombe* (Y-659) did not require inositol for growth and inhibition by lindane was very strong. *Torulaspora rosei* (Y-1567) grew better in the presence of inositol and the effect of lindane was very small. *Zygosaccharomyces acidifaciens* (Y-1011) grew equally well in the presence of lindane or inositol. A bakers yeast (Y-862) was stimulated by inositol and lindane reduced cell proliferation.

TABLE 7

Effect of lindane on lactic acid bacteria in cucumber brines, 1954

Date	Cell counts in 100,000 per ml. of brine on V-8 agar									
	1	2	3	4	5	6	7	8	9	10
7-19	1,350	1,610	1,200	1,770	1,190	2,300	1,150	1,330	1,230	3,350
7-20	740	830	660	1,040	570	1,320	930	1,860	860	2,490
7-21	130	250	120	140	180	1,750	100	490	330	1,330
7-22	52	124	68	19	49	560	107	280	141	320
7-23	137	89	70	21	176	730	69	197	147	62
7-24	30	29	17	14	82	367	46	78	67	43
7-26	12	19	6	4	20	149	27	24	49	11
Total	2,451	2,951	2,141	3,008	2,267	7,176	2,429	4,259	2,824	7,606
Mean	350	421	305	429	324	1,024	347	608	403	1,086

Analysis of variance on lactobacilli counts

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	69		
Treatment	3	910,068	8.39 **
Treat. x non-treat.	1	536,199	4.94 *
Dates	6	3,871,533	35.70 **
Replications	1	1,881,408	17.35 **
Remainder	58	108,444	

L.S.D. .05 p = 352
.01 p = 468

TABLE 8

Effect of lindane on yeasts in cucumber brines, 1954

Date	Cell counts in 10,000 per ml. of brine on AD agar									
	1	2	3	4	5	6	7	8	9	10
7-19	1,350	245	305	186	93	353	680	314	53	191
7-20	650	240	1,330	510	300	1,210	240	1,200	1,130	1,090
7-21	800	950	410	220	690	1,550	830	1,900	165	1,410
7-22	320	380	660	1,550	1,060	1,740	1,280	3,840	1,530	1,260
7-23	420	360	144	410	420	1,310	690	1,940	680	50
7-24	29	23	37	140	58	128	196	169	161	20
7-26	85	73	53	241	128	115	256	171	242	91
Total	3,654	2,271	2,939	3,257	2,749	6,406	4,172	9,534	3,961	4,112
Mean	522	324	419	465	392	915	596	1,362	565	587

Analysis of variance on yeast counts

Source	*D.F.*	*Mean Square*	*F. ratio*
Total	69		
Treatment	3	1,863,491	11.08 **
Treat. x non-treat.	1	275,879	1.64
Dates	6	2,021,565	12.02 **
Replications	1	2,535,163	15.08 **
Remainder	58	168,130	

L.S.D. .05 p = 438
.01 p = 582

TABLE 9

Lindane vs. i-inositol on the growth of yeast, bacteria and mold

Culture Number[1]	% transmittance of the cell suspension at 625 mμ							
	Lindane 17.4 p.p.m.		Lindane—17.4 Inositol—4.3		Inositol 4.3 p.p.m.		Control (water)	
Y-2007	51.0	52.5	26.0	28.5	17.0	17.5	9.0	9.0
Y-2021	82.0	84.0	82.0	80.0	64.5	67.5	66.0	69.0
Y-2240	41.5	34.0	35.0	35.0	28.5	23.0	23.0	22.5
Y-1089	77.5	76.5	50.0	44.5	26.5	28.5	35.0	37.5
Y-659	70.5	70.0	3.5	6.0	2.5	2.5	1.5	2.5
Y-1567	42.0	42.5	37.0	35.0	23.5	19.5	19.5	19.0
Y-1011	59.0	60.0	57.0	57.5	40.0	50.0	49.0	44.0
Y-862	81.5	82.5	59.0	61.0	38.0	36.5	63.0	63.5
23-2[2]	27.5	26.0	25.5	26.0	35.0	36.5	32.0	34.0
23-2[2]	0.31	0.33	0.31	0.30	0.30	0.30	0.31	0.31
G-4[3]	44.0	42.0	39.0	41.0	31.0	32.0	32.0	29.0
A-3507[4]	0.0	0.0	16.5	15.4	27.9	27.0	0.0	0.0

[1] Northern Utilization Research Branch, U.S.D.A.
[2] Department of Dairy Husbandry, University of Missouri.
[3] Percent acid as lactic.
[4] Northern Utilization Research Branch, U.S.D.A.; as milligrams of dry mycelium per fermentation flask.

Lactobacillus plantarum (23–2), the predominant lactic acid producing bacterium in cucumber fermentation, apparently does not require inositol for growth and there is indication from the data in Table 9 that lindane stimulated growth. This stimulation of growth does not appear to affect the amount of acid produced by the organism. The bacterium, *Aaerobacter aerogenes* (G-4), was not affected by either chemical, inositol or lindane; however, lindane may exhibit a slight retarding effect on growth.

The mold, *Neurospora crassa* (A-3507), which is a bioassay organism for inositol, shows a definite interaction of lindane and inositol as indicated by the data in Table 9. With inositol in the media a luxuriant mycelium mat was produced by the organism; whereas, with the same amount of inositol and a much higher concentration of lindane in the media, a much smaller amount of mycelia was produced. In the control media no growth occurred.

These findings for yeast and mold are in agreement with Buston *et al. (3)*, Kirkwood and Phillips *(11)*, and Fuller *et al. (9)*. They are not in agreement with Schopfer and Bein *(13)* and Fromageot and Cofine *(8)* who were unable to establish any relationship between lindane and inositol. Fuller *et al. (9)* working with neurospora concluded that lindane has two inhibitory effects. The first is an inhibition apparently independent of the inositol metabolism and the second is a competitive inhibition of the utilization of the growth factor by an inositol-dependent organism. In the present study, an example of the first effect is *Schizosaccharomyces pombe* (Y-659) which did not require inositol for growth, but it was inhibited very strongly by lindane. The bakers yeast (*Y-862*) and *Neurospora crassa* (A-3507) are examples of the second effect.

SUMMARY

An investigation dealing with the influence of lindane residue on the fermentation of cucumbers has been presented. Microbiological and chemical

examinations were made of the brines covering the cucumbers which were sprayed subsequent to brining to assimilate maximum residue conditions and were harvested from replicated insect control field plots for normal residue conditions. Of the pure cultures of micro-organisms studied, the yeasts and mold were affected most by lindane, and i-inositol was found to reverse the growth inhibiting effect of lindane; whereas the species of bacteria did not appear to be affected by the two compounds.

A possible scheme is proposed to explain the observed effect of lindane on the cucumber fermentation. The retardation or inhibition of the yeasts in the microflora by lindane appears to reduce the competition for the available nutrients (carbohydrates and growth factors) in the cucumber brine which the fastidious lactic acid producing bacteria require for reproduction, growth, and fermentation.

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EFFECT OF COLOR OF BEEF FAT ON FLAVOR OF STEAKS AND ROASTS

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(Manuscript received December 28, 1956)

Yellow pigment in beef fat has long been regarded as an undesirable characteristic at all levels in the marketing channel. Some people are of the opinion that yellow fat is an indication of "cow beef" or beef that is undesirable.

According to nutrition and animal husbandry personnel, the yellow coloring in fat is almost all carotene. Theories as to the source of the yellow pigment are (a) intake of large quantities of green feed or feed high in carotene, (b) older cattle which have had more time to deposit carotene in fat as a reserve, and (c) breed of cattle. Dairy breeds tend to transfer yellow coloring to their milk and to deposit it in their body fat.

In a survey of packing plants in South Carolina a wide range of price discrepancy due to yellow fat was reported. For example, a 2 cents per pound discount was frequently reported as the loss in price caused by yellow fat compared to the price of the same grade with white fat. One packer reported a loss of 8 cents per pound on a carcass which would have graded *choice*[a] except that the fat was yellow. Yellow coloring in beef fat increases the packers' marketing problems because some retailers refuse or return beef with yellow fat. Packers have learned by experience where beef with yellow fat can be sold. Sometimes the price must be reduced below that of similar beef with white fat.

In a survey of 116 adults in a citizens' education center school at Clemson, 90% reported that they paid attention to color of fat, and 73% said they would pay more for beef with fat of their color preference. Fifty-six per cent claimed to prefer white fat, 25% tinge of yellow, 10% yellow, and 9% reported no preference.

Members of home demonstration clubs in 8 representative South Carolina counties were surveyed and 79% indicated paying attention to color of beef fat and 66% said they would pay more for beef with fat of their color preference. Twenty-five pere cent claimed to prefer white fat, 28% creamy or tinge of yellow, 28% yellow, and 9% claimed no preference.

In October 1949 the color of beef fat was eliminated as a grade factor from USDA grade standards (*10*).

As a means of obtaining more objective information than that available on the effect of fat color on flavor of beef, taste panels were organized with two specific objectives as follows:

1. To determine whether a mixed group of consumers can detect differences in taste of beef steaks and roasts with yellow fat when compared to beef with white fat.

2. To determine which kind of beef, as pertains to color of fat, is preferred by more consumers when the color is masked.

[a] Refers to USDA grade. May be called USDA Choice or U. S. Choice or Choice.

PROCEDURE

Paired comparisons. Taste panels using paired comparisons seemed to be the most suitable method under the circumstances to test the effect of fat color on flavor of beef. A modification of this method was used by Cover (*1*) in testing the tenderness of meat. She called it the paired-eating method. Dawson and Dochterman (*2*) compared the paired and the triangle difference tests and concluded that the latter has some advantage when used in selecting panel members. Gridgeman (*3*) found both paired and triangle tests superior to duo-trio tests. He does not advocate adding a preference test to the pair test, however, when difference not preference *per se* is at issue. Paired samples were used by Griswold and Wharton (*4*) in studying the effects of storage conditions in palatibility of beef. Harrison and Elder (*5*) show application of statistics to several taste testing methods including paired samples. Krum (*7*) outlines detail procedure for several methods of food testing by panels. Lockart (*8*) concludes that a two-sample system should be used only for measuring quality judgment, consumer acceptance, or preference. Pilgrim and Wood (*9*) found the rating scale and paired preference method to be equally sensitive whether the difference in preference was small or large.

Samples and their selection. Samples consisting of the wholesale rib cut containing the 6th through 12th rib were used. Selection was made by a cooler salesman and an agricultural economist. Two carcasses, alike in visible characteristics except for a difference in fat color, were selected for each comparison. For example, carcasses were of similar cooler aging from animals of the same grade, weight, and estimated age. Sex was the same for each pair except that 109 was a heifer and 110 a steer.

Grades of meat. Three pairs of samples graded *commercial*, 2 *good* and 1 *choice*.

Retail cuts. From 4 to 7 rib steaks about ½ inch thick were cut, thus leaving from 4 to 6 ribs together as a standing rib roast. Care was taken to obtain steaks and roasts as nearly the same thickness and weight as possible from the two carcasses being compared.

Cooking. Pairs of steaks were broiled alike until medium-well or well done. Roasts were cooked for 30 minutes per pound at oven temperature of 149° C. (300° F.) and were kept hot for serving. Pieces of meat to be compared were selected and cooked as nearly alike as possible. No seasoning was used either in cooking or in tasting. Electric ovens were used for steaks and roasts except that it was necessary to use a gas oven to start the roasts on several occasions while the electric ovens were being used with steak panels.

Instructions to panel members. Instructions to panel members were held to a minimum in an effort to avoid giving suggestions. Some panel members were told that 2 colors of fat were being tested. Instructions were of the following nature: You will be given 8 samples of steak (roast). We want you to decide whether the samples are alike or different. That will be 4 comparisons. Some samples will be alike. We are mainly interested in taste or flavor. Please do not eat crackers with the meat. The code number will be the two numbers on cards before you.

Cutting samples for paired comparison. One of the main difficulties encountered in conducting the taste panels was caused by the lack of homogeneity of the meat. One person did all the cutting and placing the samples on saucers to reduce bias caused by differences within and between muscles of meat. The portion of one sample was compared with the identical portion of the other sample. When a sample from the same carcass was placed on the two saucers for comparison, similar care was exercised to get 2 samples as much alike in every way as possible.

Serving. A bite size cut with fat removed was placed on each of 2 saucers and served in pairs. Table knives and forks and saltine crackers were placed in the 6 booths. Each booth was provided with running water.

All cutting platters and serving saucers were heated in an oven so that samples would be warm for the panels. Panel members or judges were seated in 6 individual booths in a room separated from but adjoining the kitchen where meat was cooked and cut and dishes were washed. The round-table method (*3*) was not used in any comparisons.

Generally, all judges were seated; then instructions were given. Small serving trays were employed, and a pair of samples with identifying numbers could be carried on each tray. Red lighting was used in the booths in an effort to make all samples appear alike

in color. "Walking around" was negligible, reduced to that degree by cutting out overhead lights in the panel room and by having panel members arrive at an appointed time or wait for their turn in a room across the hall from the panel room on the few occasions when they came early. It was found that cutting samples, serving, and keeping the reports in order were facilitated by having all panel members taste the same pair of samples simultaneously.

Reports. Reports were made by each panel member for each comparison on the following form:

Product **Beef** **Roast** **Steak**
Circle one sampled

Paired Comparison

Code_____

Name_____ Date_____

Two samples are before you. They may be the same or different. Please decide if they are the same or different.

(1) Are the samples different? Yes_____ No_____

(2) If different, which do you prefer?_____

Comments (reason for preference)_____

Selection of panels and number participating. It was decided that for a preference study the opinions of a large number of individuals would be more valuable than the opinions of a trained panel; therefore, untrained judges were used *(6)*. A total of 189 different individuals participated as panel members. Some served more than once. For example, 1 served 5 times, 3 served 4 times, 7 served 3 times, and 28 served twice. Counting repeaters a total of 244 served, giving a yield of 976 comparisons.

Time of tasting. The panels served during the weeks beginning May 7 and 14, 1956. Actual tasting was done on 8 different days. Steaks were compared during the mornings and roasts were compared during afternoons. Hours of test were usually 9:30 to 11 A.M. and 2-4 P.M.

Coding samples. Every precaution was taken to keep panel members from figuring out a system of comparisons of meat being served. Samples were always served in pairs and consecutive numbers were used. For example, the first comparison was numbered 1 and 2, the second 3 and 4, etc. Letters A and B were used by the meat cutter. For example, A was for yellow fat and B for white fat. When meat with different colored fat was used the order of numbering samples was varied by assigning the lower number (odd number) to yellow fat meat for one comparison and to white fat meat for the other comparison. Odd numbers were always to the taster's left.

RESULTS

Steaks. Tasters were asked whether there was a difference between the 2 samples before them, and if so, which they preferred. Pilgrim and Wood *(9)* used a similar method for preference testing. For two of the 4 comparisons, one sample was from yellow fat beef, and one was from white fat beef. For the other 2 comparisons both samples were from the same piece of meat.

A highly significant number, as calculated by the Chi Square test,[b] of the

[b] Formula and general idea for procedure were obtained from a report by Krum *(7)*. Formula used to test significance was:

$$X^2 = \frac{[(X_1 - X_2) - 0.5]^2}{N}$$

Where X = individual score or measurement
X_1 = number correct (the larger number)
X_2 = number incorrect (the smaller number)
N = number of tasters

TABLE 1

Steaks: comparison of steaks with yellow fat to those with white fat. Yellow-white arrangement, taste panels, Clemson College, May, 1956.

Sample numbers	Grade	Number of tasters	Difference observed		Preference[1]	
			Yes	No	Y	W
109–110	G	17	13*	4	8	5
113–114	C	18	14*	4	9	5
115–116	C	16	11	5	6	5
117–118	C	18	14*	4	2	12**
119–120	G	18	12	6	2	10*
121–122	Ch	18	12	7	4	8
Total	....	105	76**	29	31	45
Percent of total	....	100	72	28	30	43

[1] Y = Yellow fat, W = White fat.
* Significant at 5% level.
** Significant at 1% level.

total panel members observed a difference between the yellow and white fat presented them, Table 1. All comparisons shown in Table 1 include both yellow and white fat beef. There appeared to be a preference trend for beef with white fat; however, 45 out of the 76 cases observing a difference would not prove to be significant at the 5 per cent level. That means that in 5 tests of 100, as many as 45 tasters could have indicated a preference for beef with white fat merely by chance alone or by guessing without tasting. For 2 pairs of samples (117-118 and 119-120) a significant number of those detecting a difference expressed a preference for white fat steaks. Elimination of those who did not observe a difference was justified by the Chi Square test of independence.[e] Dawson and Dochterman (2) advocate the use of untrained panels for preference studies.

When the order of arrangement of the 2 samples was changed so that the white fat beef was to the left of the panel member and the yellow fat to the right, 55 of the 76 observing a difference between the 2 samples indicated a preference for steak with white fat, Table 2. This indicates a preference at the 1% level of significance for beef with white fat for those who observed a difference. A significant number of panel members tasting steak pairs 119-120 and 121–122 expressed a preference for beef with white fat on the basis of the total number tasting those samples.

Comparing the total number in each sample preferring steak with different colors of fat, the majority preferred yellow fat 3 times, while white fat was preferred 9 times out of the 12 comparisons shown in Tables 1 and 2.

By combining results from two arrangements of pairs of samples for all panel members when steaks with different colors of fat were used, the effect of obtaining responses from twice as many tasters is obtained. Such combining

[e] Analysis by the Chi Square test of independence indicates that a significant number detected a difference when steaks with different colored fat were tasted, as compared to the check samples when steaks from the same carcass were sampled. Thus, it is highly unlikely that as many as detected a difference could have done so by chance alone. That many tasters would not have indicated a detection of difference 1 time out of a hundred purely by chance alone or by guessing.

TABLE 2

Steaks: comparison of steaks with white fat to those with yellow fat. White-yellow arrangement.

Sample numbers	Grade	Number of tasters	Difference observed		Preference[1]	
			Yes	No	Y	W
109–110	G	17	10	7	4	6
113–114	C	18	12	6	3	9
115–116	C	16	12*	4	2	8
117–118	C	18	9	9	1	7
119–120	G	18	17**	1	4	13*
121–122	Ch	18	16**	2	4	12*
Totals	....	105	76**	29	18	55**

[1] Y = Yellow, W = White.
* Significant at 5% level.
** Significant at 1% level.

seems permissible since the same two animals were involved, but the arrangement of the 2 samples in front of the tasters was reversed. The results of combining Tables 1 and 2 are shown in Table 3 where 210 comparisons were made. There was a highly significant proportion of those who observed a difference indicating a preference for white fat steaks. Of the 149 expressing a preference, 100 preferred white fat steaks.

An inspection of separate pairs reveals that there was a significant proportion of those detecting a difference expressing a preference for white fat steak in 3 of the 6 pairs. In 2 of these 3 cases a significant number had detected a difference and expressed a preference.

As a means of checking on the taste perception of panel members, 2 of the 4 paired comparisons given each taster were taken from the same carcass. There was no case in which a significant number of panel members detected a difference between the 2 samples when considered from the standpoint of individual samples or when considered as a total of the particular pair, Table 4. It appears that some judges were guessing that there was a difference since one-half of them reported a difference when comparing cuts from the same steak. When the 2 groups of pairs yellow-yellow and white-white were com-

TABLE 3

Steaks: combining responses[1] for two arrangements (yellow-white and white-yellow) of paired comparisons for steak with different colors of fat

Sample numbers	Grade	Number of tasters	Difference observed		Preference[2]	
			Yes	No	Y	W
109–110	G	33	22	11	12	11
113–114	C	36	26**	10	12	14
115–116	C	32	23*	9	8	13
117–118	C	36	23	13	3	19**
119–120	G	36	29**	7	6	23**
121–122	Ch	36	28**	8	8	20*
Totals	....	209	151**	58	49	100**

[1] Combining Tables 1 and 2.
[2] Y = Yellow, W = White.
* Significant at 5% level.
** Significant at 1% level.

TABLE 4

Steaks: combining responses for two comparisons of steak from the same carcass. Taste panels, Clemson College, May, 1956.

Sample numbers	Grade	Number of tasters	Difference observed		Preference	
			Yes	No	Odd	Even
109–110	G	34	18	16	9	7
113–114	C	36	19	17	7	11
115–116	C	32	15	17	6	9
117–118	C	36	18	18	6	12
119–120	G	36	17	19	9	8
121–122	Ch	36	18	18	9	9
Totals	....	210	105	105	46	56

bined, there was still not a significant number detecting a difference between the samples. Likewise, there was no significant tendency to prefer the odd or even numbers.

On the basis of the results from the 76 of the 105 tasters who observed a difference, there appears to be a preference for steaks from beef with white fat for the samples used. More samples need to be compared before definite conclusions can be reached.

Roasts. Roasts from the same animals from which steaks were obtained were tested using the same techniques that were used for steaks. Of course, preparation of the steaks and roasts was different. A larger number of tasters was involved with the roasts than with the steaks because of the relative ease of preparing and serving samples of roasts. A total of 139 tasters served on the roast panels. When yellow and white fat roasts were compared, a highly significant number of tasters observed a difference between the 2 samples in both arrangements, i.e., yellow fat beef to left, Table 5, and white fat beef to left, table 6. A signfiicant number of panel members comparing samples number 109 and 110 and those comparing 117 and 118 detected a difference between the meat from the 2 animals for both steaks and roasts.

When the white fat roast was at the taster's left, a signfiicant proportion of the judges in 3 of the 6 pairs observed a difference between the white and

TABLE 5

Roasts: comparison of roasts with yellow fat to those with white fat. Yellow-white arrangement.

Sample numbers	Grade	Number of tasters	Difference		Preference[1]	
			Yes	No	Y	W
109–110	G	15	14**	1	8	6
113–114	C	16	11	5	6	5
115–116	C	24	20**	4	10	10
117–118	C	24	17*	7	8	8
119–120	G	30	18	12	9	9
121–122	Ch	30	12	18	5	7
Totals	....	139	92**	47	46	45
Percent of totals	....	100	66	34	33	32

[1] Y = Yellow, W = White.
* Significant at 5% level.
** Significant at 1% level.

TABLE 6

Roasts: comparison of roast with white fat to those with yellow fat. White-yellow arrangement.

Sample numbers	Grade	Number of tasters	Difference		Preference[1]	
			Yes	No	Y	W
109–110	G	15	13**	2	0	13**
113–114	C	16	11	5	8	2
115–116	C	24	17*	7	9	7
117–118	C	24	15	9	9	5
119–120	G	30	18	12	11	7
121–122	Ch	30	27**	3	17	10
Totals	....	139	101**	38	54	44
Percent of totals	....	100	73	26	39	32

[1] Y = Yellow, W = White.
* Significant at 5% level.
** Significant at 1% level.

yellow fat roasts, Table 6. As was true for sample pair 109 and 110 in the first group of yellow-white arrangement, it was also true for the white-yellow arrangement that a highly significant number of judges reported a difference between the 2 samples. There was a highly significant number preferring white fat roast in that pair of samples only. In no other comparison was there a unanimous preference for either fat color roast or steak. There was no other significant preference expressed for either white or yellow fat roast.

A combination of Tables 5 and 6 gives the equivalent of 278 taste comparisons for roast with yellow fat and white fat, Table 7. This combination shows that a highly significant number of tasters detected a difference between roasts with different colors of fat, but there was no significant preference for roast with either yellow or white fat.

As a means of checking the taste perception of panel members as was done with steaks, 2 pairs of similar samples (one pair from the yellow fat roast and one from the white fat roast) were given tasters. With a total of 278 tastings, no significant difference between samples was detected, Table 8. There was also no significant number preferring any sample. A significant number of

TABLE 7

Roasts: combining responses for two arrangements of paired comparison for beef roast with different colors of fat[1]

Sample numbers	Grade	Number of tasters	Difference		Preference[2]	
			Yes	No	Y	W
109–110	G	30	27**	3	8	19
113–114	C	32	22*	10	14	7
115–116	C	48	37**	11	19	17
117–118	C	48	32*	16	17	13
119–120	G	60	36	24	20	16
121–122	Ch	60	39*	21	22	17
Totals	....	278	193**	85	100	89

[1] Combining Tables 5 and 6.
[2] Y = Yellow, W = White.
* Significant at 5% level.
** Significant at 1% level.

TABLE 8

Roasts: combining responses for two comparisons of roast from the same carcass

Sample numbers	Grade	Number of tasters	Difference		Preference	
			Yes	No	Odd	Even
109–110	G	30	14	16	4	10
113–114	C	32	16	16	7	8
115–116	C	48	21	27	9	12
117–118	C	48	21	25	13	8
119–120	G	60	35	25	13	18
121–122	Ch	60	28	32	9	19
Totals	....	278	135	141	55	75

tasters comparing 2 yellow fat samples from sample 119 reported a difference; however, the same panel members were about equally divided as to preference for the 2 samples being compared, thus not showing a preference for either over the other. This is not shown in the table. Here again, as in the steak sampling, it appears that some judges were guessing that there was a difference.

SUMMARY AND CONCLUSIONS

Steaks and roasts were tasted by taste panels to determine whether there was a taste difference associated with color of beef fat, and if so which fat color was associated with the preferred meat. Rib steaks and rib roasts from the same wholesale cut were used. Carcasses from which samples were obtained were selected so that meat with yellow fat was as much as visibly possible like meat with white fat except for fat color. Panel members were untrained and were obtained from staff, faculty, stenographic workers and students of Clemson College. A total of 105 tasters or judges sampled steaks and 139 sampled roasts, giving a total of 244. Some judges served more than once; therefore, reducing the number of different individuals to 189. For the samples from the 12 carcasses selected for comparison the following conclusions were reached:

1. A significant number of judges for beef steak and roast detected a difference in the taste of beef with yellow fat and that with white fat.

2. When sample arrangements yellow-white and white-yellow were combined giving a total of 209 tastings, there was a highly significant number of tasters who detected a difference in taste of steak with yellow fat as compared with steak with white fat. There was also a highly significant proportion expressing a preference for steak with white fat among tasters who detected a difference in taste. Analysis of results from control samples justified eliminating from analysis those who did not detect a difference when yellow and white fat samples were tasted.

3. On 2 of the 4 comparisons made by each panel member, steak from the same animal was served. Where these two comparisons were combined, the 210 tastings showed no significant difference in taste and no significant preference for either sample. These results strengthen results given when different colors of fat were used in which significant numbers detected a difference.

4. There was no flavor preference indicated for roast beef on the basis of color of fat, although a significant number of tasters observed a difference between samples when fat color was different.

5. Further tasting will be necessary before firm conclusions can be drawn.

Acknowledgments

The author is indebted to Dr. D. M. Graham, Associate in Dairying and Assistant Professor of Dairying, Clemson College and to L. O. Van Blaricom, Associate Food Technologist and Associate Professor of Food Technology, Clemson College, for their assistance in planning details of setting up the taste panels, and to Mrs. Mary Jane Marbut, Assistant Nutritionist, Nutrition Department, Clemson College, for cooking samples and assisting with kitchen supervision, and to Dr. G. H. Aull, Head, Department of Agricultural Economics and Rural Sociology, Clemson College, Clemson, South Carolina, for his advice and general supervision of the experiment. The author is also grateful to those who acted as judges on the taste panels and to those who helped serve the samples to panel members.

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DETERMINING FLAVOR DIFFERENCES IN CROPS TREATED WITH PESTICIDES. I. A COMPARISON OF A TRIANGLE AND A MULTIPLE COMPARISON METHOD[a]

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(Manuscript received December 31, 1956)

Taste testing has become an accepted tool for determining whether or not pesticides impart off-flavors to foods. The methods of conducting these tests include paired tests, triangle tests, and scoring tests. Paired comparison or triangle methods are often assumed to be more exact than scoring methods, but a number of research workers have used some form of scoring because it is convenient or economical *(4)*.

Peryam and Swartz *(3)* state that "tests which depend upon discrimination rather than upon judgments give greater objectivity." Hening *(1)* considered the triangle method to be a very satisfactory discriminative test. Although these views suggest that either a paired comparison test or a triangle test is more precise than a scoring test, the experiments which have actually compared methods do not bear this out *(2, 4)*. Pilgrim and Wood *(4)* studied paired comparison and rating scale methods for detecting differences in preference and reported both methods to be equally sensitive whether the differences in preference were small or large. Kramer and Ditman *(2)* compared the triangle test and a rating scale method, referred to as multiple comparison. They concluded that the multiple comparison was superior to the triangle method in determining flavor differences. The multiple comparison was also regarded as superior because it gave additional information about the direction and importance of differences.

In this laboratory a multiple comparison and a modified triangle test were compared to determine which one was more precise, the relative amounts of samples needed, and how much time was used by the operator and the judges.

EXPERIMENTAL

Samples. The materials used in the comparisons were:

Green beans grown and canned as one-inch cuts at the University of Maryland, College Park, Maryland. There were six samples, one from untreated plants (check or standard) and one each from plants treated with pesticides, as follows: malathion, one application early; malathion, one application late; malathion, one appliction early and one late; dilan, one application early; dilan, one application early and one late.

Squash grown at the Agricultural Experiment Station at Waltham and canned as purée at the University of Massachusetts, Amherst, Massachusetts. There were four treatments, as follows: 7% captan (check or standard); 4% malathion plus 7% captan; 0.25% lindane plus 7% captan; and 2% endrin plus 7% captan.

For each session the contents of sufficient cans of each treatment were combined. The beans were chopped into small pieces. One ounce of each treatment was served in

[a] Contribution of the Northeast Regional Cooperative Project, NE-15. Effect of Pesticides on Quality of Fruits and Vegetables, and supported in part by regional funds of the United States Department of Agriculture and by State funds.

a 3-oz. crystallizing dish. Dishes were coded with letters selected at random and then set in a pan of hot water for from 10 minutes to 15 minutes before serving so that they could be judged at a temperature similar to the temperature at which green beans and squash are usually served.

Taste tests. The score sheets for the triangle and the multiple comparison tests are illustrated in Figures 1 and 2. Because there was a limited amount of standard material for the beans, the odd sample in the triangle was the standard or check. Each of 15 judges was given at random at each session 2 of the 3 possible triangle arrangements with the restriction that the 3 possible arrangements appeared 10 times each session. With the squash each judge was given at random at one session 2 of the 3 triangles in which the standard was the odd sample, at another session 2 of the 3 triangles in which a treated sample was the odd sample, with the restriction that the 3 possible arrangements appeared 10 times each session. The multiple comparison consisted of 6 samples of beans or 4 of squash, one of each treatment including the check. A marked check sample was presented for use as a reference standard. The order of presentation of the samples was randomized.

TRIANGLE
FLAVOR DIFFERENCE EVALUATION
Judge's Score Sheet [1]

Date_____ Product_____

Project_____ Treatment or Can Code_____ Panel Replicate_____

Name of Judge_____ Code No. or Letter for Judge_____

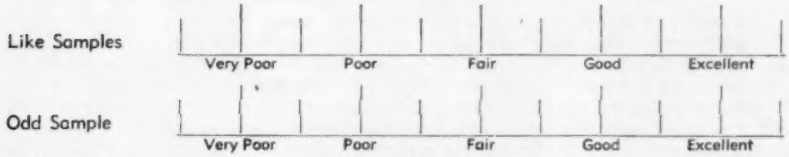
Separate for Flavor Only

Sample	Check Like Samples*	Flavor Difference between odd and like samples		Did you check by guess?
(1)	(2)	(3)		(4)
_____	_____	____None	____Slight	Yes____
_____	_____			
_____	_____	____Moderate	____Much	No____

* Check by guess if no difference is detectable.

5. If you checked a Moderate or Much in the flavor difference column (No. 3), then indicate below whether you consider either the odd sample or like samples to have an undesirable flavor.
   Odd Sample: Yes____ No____ Like Samples: Yes____ No____

6. **Rate for Flavor Only.** Place a check mark above either a short or long line on the scale below to indicate how you rate the like samples and the odd sample.



7. (a) Did you detect any kind of difference, other than flavor, between the samples?
   Yes____ No____

   (b) If "Yes," what kind of difference?_____

[1] This score sheet was originally prepared and used by a group of cooperators in a study coordinated by the National Canners Association, Washington, D.C.

Figure 1. Score sheet for triangle method of flavor difference evaluation.

MULTIPLE COMPARISON

FLAVOR EVALUATION

Rate Flavor Only. Ignore All Other Differences.[1]

Project NE-15_____

Product_____

Date: Code_____

Name:

Trial A	Better than average in flavor	Average in flavor (Similar to standard)	Below average in flavor—no distinguishable off-flavor	Slightly off-flavor	Definitely off-flavor—not acceptable
Sample 1					
2					
3					
4					
5					
etc.					

[1] Verbal instruction was to compare each sample with the marked "standard" sample.

Figure 2. Score sheet for multiple comparison method of flavor evaluation.

Because of the limited amounts of check sample of beans, only 2 replications of each method were obtained, necessitating 5 sessions for triangle and two for multiple comparison tests. For the squash, 4 replications of each method were obtained, necessitating 6 sessions for triangle and four for multiple comparison tests.

Panel selection. A panel of 15 tasters for beans and a panel of 15 tasters for squash were selected from 21 persons who volunteered to serve as judges. Ten persons served on both panels. Triangle tests were used to select the judges. To select the panel to test the bean samples, 2 different brands of commercial beans were used because there was a limited amount of the experimental beans. To select the panel to test the squash, the control and a treated sample were used. For each vegetable the 15 persons who had the most correct separations in 3 triangles were chosen.

RESULTS AND DISCUSSION

The results of the triangle tests are shown in Table 1 and and of the multiple comparison tests in Table 2.

With the beans, the results for the triangle and multitple comparison did not agree, but the results of both tests indicated that no treatments had truly off-flavors. In the triangle test the number of correct separations indicated that samples treated with malathion one application late and dilan one application early were significantly different from the check. These differences were probably not due to off-flavors because the treatments were not significantly different from the check in the multiple comparison test and the flavor rating scores indicated the treated samples were better than the check. The differences in flavor rating scores, even though statistically significant, were very small and probably of no practical significance. In the multiple comparison test the only treatment that was significantly different from the check ($.05 \geqslant P \geqslant .01$) was malathion one application early and the difference was observed only by the more discriminating judges, i.e., when the scores for the

TABLE 1
Triangle tests

Treatment compared with standard	Total number of judgments	Number of correct separations	Number of guesses		Flavor rating scores			
					For all judgments		For samples correctly separated	
					Average ± Std. Dev.		Average ± Std. Dev.	
			Right	Wrong	Standard	Treatment	Standard	Treatment
Beans								
Malathion (1 application early)	30[1]	12	4	6	5.8 ± 0.3	6.3 ± 0.2	5.0 ± 0.4	5.0 ± 0.6
Malathion (1 application late)	30	24**	..	3	5.0 ± 0.3	6.0 ± 0.2[2]	5.0 ± 0.4	6.2 ± 0.4[3]
Malathion (1 application early and 1 late)	30	13	8	8	6.0 ± 0.3	6.1 ± 0.3	5.8 ± 0.5	6.4 ± 0.5
Dilan (1 application early)	30	22**	2	1	4.6 ± 0.4	6.3 ± 0.2[3]	4.1 ± 0.4	6.4 ± 0.3[3]
Dilan (1 application early and 1 late)	30[1]	3	..	6	5.6 ± 0.4	5.5 ± 0.3	6.7 ± 0.7	7.4 ± 0.4
Squash								
Lindane plus Captan	60	24	4	13	3.5 ± 0.2	3.4 ± 0.2	3.7 ± 0.4	3.9 ± 0.3
Malathion plus Captan	60	38**	6	5	3.2 ± 0.2	3.8 ± 0.2	2.9 ± 0.3	3.9 ± 0.3[3]
Endrin plus Captan	60	32**	7	7	3.7 ± 0.2	3.3 ± 0.2	4.3 ± 0.3	3.7 ± 0.4[3]

** $P \leq .01$.
[1] One substitute judge.
[2] Treatment different from standard $.05 \leq P \geq .01$ using *t* test *(6)*.
[3] Treatment different from standard $P \leq .01$ using *t* test. *(6)*.

TABLE 2

Multiple comparison tests

	Average scores		Analyses of variance			
			Mean squares—treated versus standard			
	All judges	Selected judges [1]	All judges		Selected judges	
			d.f.	mean sq.	d.f.	mean sq.
Beans						
Standard (untreated)	0.13	0.83	..	....	....	....
Malathion (1 application early)	0.00	−0.08	1	<1	1	5.04*
Malathion (1 application late)	0.47	0.75	1	1.67	1	<1
Malathion (1 application early and 1 late)	0.60	0.25	1	3.27	1	2.04
Dilan (1 application early)	−0.07	0.17	1	<1	1	2.67
Dilan (1 application early and 1 late)	0.47	0.58	1	1.67	1	<1
			Judge 60 x treatment interaction	1.10	25	1.03
Squash						
Standard Captan	0.39	1.05	..	....	....	....
Lindane plus Captan	0.57	0.60	1	<1	1	2.02
Malathion plus Captan	0.21	0.35	1	<1	1	4.90
Endrin plus Captan	−0.80	−0.90	1	40.68**	1	38.02**
			Judge 39 x treatment interaction	2.47	12	2.70

[1] Omitting the scores for 9 judges who rated the unnown standard as "off-flavor" one or more times.
* $.05 \geqq P \geqq .01$.
** $P \geqq .01$.

9 judges who rated an unknown check as "off-flavor" were eliminated from the analysis. The fact that malathion one application early was significantly different ($.05 \geqslant P \geqslant .01$) to only these 6 judges indicates that there might have been a slight off-flavor in this treatment but this off-flavor was evident to a discriminating few.

With the squash, the results for the triangle and multiple comparison tests showed some agreement. In the triangle test the number of correct separations and the flavor ratings indicated that the samples treated with malathion-captan were significantly better and with endrin-captan were significantly poorer than the check ($P \geqslant .01$). In the multiple comparison test endrin-captan was rated significantly poorer than the check ($P \geqslant .01$). The conclusion was made that the endrin-captan treated squash had an off-flavor.

Lack of agreement between the results for the triangle and multiple comparison tests may have been caused by several factors. For example, if there was a difference in flavor of samples the triangle test would result in enough correct separations to indicate a significant difference, but in the multiple comparison test, if the difference in flavor was not an off-flavor the judges could indicate only a preference (see Figure 1). If some of the judges rated

a sample as better than check and some rated it as poorer than check, the scores would tend to balance each other and the panel scores for treated and check would not be significantly different. This situation might explain why the beans treated with malathion one application late or dilan one application early and the squash treated with malathion-captan were significantly different from check in the triangle test but not in the multiple comparison test. Another factor that could have caused lack of agreement was that, in the triangle tests, slight differences in texture or color may have influenced the judges to make correct separations. This effect of such slight difference was less likely to influence the judges in the multiple comparison test. For example in the triangle tests with green beans judges indicated 14 times a difference in texture among samples and half of the time a texture difference was indicated the separations were correct. In the squash judges indicated 3 times a difference in color between malathion-captan and the check and each time this difference was indicated the separations were correct.

One advantage that Kramer and Ditman *(2)* ascribed to the multiple comparison was that the test would show direction and size of flavor differences. The triangle test used in this experiment was modified by the addition of a flavor rating scale to show direction and size of differences. By this scale in the triangle test the ranking of the samples relative to the check was different from the ranking by the mean scores in the multiple comparison test. Because the scores depended partially upon the preferences of the judges, the disagreement in ranking could have been due to changes in judges' preferences either with lapse of time or with differences in manner of indicating preferences (see Figures 1 and 2).

One possible explanation for the bean samples rating better than check in the triangle and poorer than check in the multiple comparison might be that, due to the limited amount of check sample, the odd sample in the triangle was always check. Schultz *(5)* reported a definite bias against the odd sample in triangle tests resulting in the odd sample being rated lower than the like samples when little or no difference existed.

Average time per session required by the operator for conducting the triangle test (Table 3) differed only slgihtly from that for the multiple comparison tests, but triangle tests occupied more sessions than multiple comparison tests, three more on the beans and two more on the squash. The judges took slightly more time to taste a triangle set than a multiple comparison.

With the beans, the triangle tests required 20 cans of the check or standard and 9 cans of each treated sample, whereas the multiple comparison tests

TABLE 3

Average time for operator and judges

	Number of sessions	OPERATOR Minutes per session	JUDGE Minutes per session
Green Beans			
Triangle	5	174	7.6
Multiple Comparison	2	187	6.0
Squash			
Triangle	6	150	5.5
Multiple Comparison	4	132	5.3

required only 8 cans of the check or standard and 4 of each treated sample. With the squash, the triangle tests required 27 cans of the check or standard and 9 cans of each treated sample, whereas the multiple comparison tests required only 16 cans of check or standard and 8 cans of each treated sample.

SUMMARY AND CONCLUSIONS

Two methods used to evaluate flavor differences in foods, triangle and multiple comparison, were compared using samples from green beans and squash plants which had been treated with pesticides. The judges were selected on their ability to differentiate between samples rather than on their ability to evaluate. The findings by the two methods with the beans did not agree. In the triangle tests the malathion one application late and dilan one application early were significantly better than the standard. In the multiple comparison test the sample malathion one application early was rated significantly poorer. No other differences were apparent from these tests. With the squash both tests indicated that the endrin-captain sample was off-flavor and the triangle test also indicated that the malathion-captan sample had a flavor better than the standard. The multiple comparison test took less time and less samples than the triangle test.

From these results the conclusion is made that the only sample which had an off-flavor, squash treated with endrin-captan, was picked out by both tests. The multiple comparison method was more desirable because it took less time, less samples and small differences in color and texture apparently did not influence the results. The triangle method was more precise because it indicated differences that the multiple comparison did not show, but these differences were not necessarily off-flavors.

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WHEAT STARCH PASTES AND GELS CONTAINING CITRIC ACID AND SUCROSE

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(Manuscript received January 28, 1957)

In much of the review literature on starch, the property of gel formation is ascribed entirely to amylose, the linear component of starch. Clear distinction has not always been made between the properties of starch pastes on the one hand and of solutions of amylose and amylopectin on the other. According to Meyer *(5)* amylose is not sufficiently soluble in water to make possible the preparation of aqueous solutions concentrated enough to form gels. Some evidence has been obtained to support the theory that amylose in solution is largely responsible for rigidity of cooled starch pastes *(2, 3)*. Other evidence appears to be in conflict with this theory *(2)*.

Another relationship which warranted further study is that between granule size and the properties of starch pastes and gels. The literature contains relatively little information *(6, 7, 8)*.

This investigation was undertaken to obtain further evidence concerning the above relationships and also to study the effects of citric acid and of sucrose on the properties of the pastes and gels of wheat starch. The fact that unexpected results sometimes are obtained in the preparation of foods thickened with starch suggests the existence of interactions between different ingredients and the desirability of studying several factors simultaneously.

EXPERIMENTAL

Materials and preparation of pastes. Starch was washed from doughs of hard wheat flour, bleached [b] and unbleached,[c] and soft wheat unbleached flour.[d] Approximately one-half of the soft wheat starch was bleached [e] after extraction. Each of the 4 lots of starch [f] was sized into 3 fractions by a sedimentation procedure. Large-granule fractions consisted of the granules which in one hour settled out of a suspension of starch and water in a 33-cm. column. Medium-granule fractions consisted of the granules which settled out of the remaining suspension in 95 minutes. Small-granule fractions consisted of the granules which did not settle in 95 minutes and were removed from the suspension by centrifugation. All fractions were air-dried and passed through a 40-mesh sieve. Approximately one-fourth of each of the 4 original starches was left unfractionated.

In addition to the 4 sources of starch and 4 granule-size distributions, 4 concentrations of citric acid were used, 0, 0.050, 0.100 and 0.200 N, and 4 concentrations of sucrose, 0, 10, 20 and 40 g. per 100 ml. water or citric acid solution. The 256 treatments resulting from all possible combinations of the 4 factors, each at 4 levels, were arranged in a manner permitting statistical evaluation of the effect of replication.

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[a] Present address: Department of Home Economics, University of California at Los Angeles.

[b] Purchased on the local market.

[c] Milled at the Urban Mills, Buffalo, New York, from wheat of the Cheyenne variety, grown in Wyoming in 1953.

[d] Milled at the Urban Mills from wheat of the Genesee variety grown in New York state in 1954.

[e] Bleached at the Urban Mills: Beta chlora, one ounce per 100 lb.

[f] Hereafter referred to as sources of starch.

Pastes were prepared in a Brabender amylograph in which the standard spring had been replaced with a 1.1-mm. spring. The cooling coil was not placed in the suspensions. Heating was begun at 25° C. and continued to 93° C., at which temperature the pastes were held for 15 minutes. At the end of the heating period two aliquots were taken from each of the pastes except those containing unfractionated starch or the second highest concentration of sucrose or the second highest concentration of acid. An aliquot of approximately 5 g. was diluted with water to a volume of 50 ml. for granule measurements and photomicrographs. A weighed aliquot of approximately 50 g. was suspended in 50 ml. of water for estimation of iodine uptake by the amylose in solution. Portions of all pastes were poured into jelly glasses 36–37 mm. in depth, covered and stored in a constant temperature oven at 30° C. for approximately 24 hours.

Measurements. Maximum viscosity, viscosity when the paste temperature reached 93° C. and viscosity at the end of the heating period were read from the amylograms. Viscosity at the end of the heating period hereafter will be referred to as terminal viscosity.

A Precision penetrometer was used for measuring the sag of gels unmolded after storage. Depth of the jelly glass was assumed to be the original height of the gel in the calculation of percent sag.

The colorimetric procedure of McCready and Hassid *(4)*, as modified by Hester *et al. (3)*, was used for measuring the uptake of iodine by the amylose in solution. The suspended aliquot was centrifuged and a dilute solution of the supernatant was titrated with a dilute iodine solution. Iodine uptake was calculated per gram of dry weight of starch.

Granule-size distributions were obtained for those pastes for which iodine uptake was measured. A drop of dilute suspension was placed on a hemocytometer and the long axis of each granule in the 1-mm. center square was measured under the microscope by means of an ocular micrometer. A magnification of 430X was used. When granules were very small and numbered more than 500 in the center square, only 500 were measured. When severe fragmentation of granules made measurement extremely difficult, some estimating was necessary.

All of the methods described are given in greater detail in the study by Campbell *(1)*.

RESULTS

Temperature of gelatinization. In general, pastes of large-granule fractions began to thicken at lower temperatures and attained maximum viscosity at lower temperatures than did pastes of small-granule fractions (Figure 1).

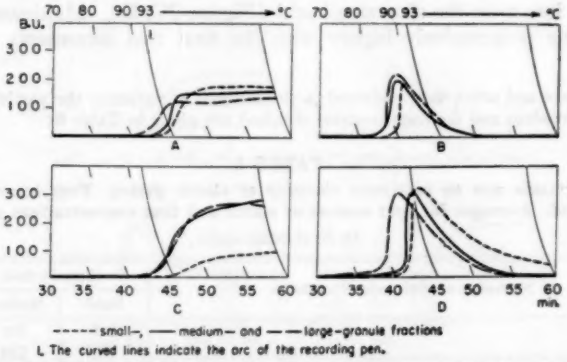

Figure 1. Amylograms illustrating the effect of granule size on viscosity-temperature curves of starch pastes. Starch from soft wheat unbleached flour. Four combinations of citric acid and sucrose concentrations: (A) No citric acid, no sucrose. (B) 0.200 N citric acid solution, no sucrose. (C) No citric acid, 40 g. sucrose/100 ml. water. (D) 0.200 N citric acid solution, 40 g. sucrose/100 ml. citric acid solution.

TABLE 1

Effect of granule size on maximum viscosity of starch pastes in the absence of citric acid and sucrose. Four sources of starch

In Brabender units

Source of starch	Granule-sized fraction		
	Small	Medium	Large
	BU	*BU*	*BU*
Soft wheat unbleached	180	164	132
Soft wheat bleached	250	160	143
Hard wheat unbleached	80	88	97
Hard wheat bleached	150	120	120
Average	165	133	123

Citric acid lowered the temperatures required for gelatinization, as may be seen in Figure 2 by a comparison of the individual curves in C and in D or by a comparison of the set of curves in A with the set in B. The general effect of sucrose was a delay in gelatinization, as is illustrated in Figure 2 by a comparison of the individual curves in A and in B or the set of curves in C with the set in D.

Viscosity of pastes.[5] Maximum viscosity became lower with increasing granule size in the absence of citric acid and sucrose (Table 1). In the presence of sucrose (Table 2), maximum viscosity became higher with increasing granule size at all concentrations of citric acid. In citric acid solutions (Table 3), maximum viscosity of pastes was unaffected by granule size in the absence of sucrose and increased with increasing granule size in the presence of sucrose. The effect of granule size became greater as the concentration of sucrose was increased. Granule size affected viscosity at 93° C. and terminal viscosity similarly to maximum viscosity, the effect on terminal viscosity being particularly marked.

Maximum viscosity became considerably higher with the first increment of citric acid. With further increments of acid, it became progressively lower but not as low as in the absence of acid (Figure 2C, D). Maximum viscosity also became progressively higher with the first two increments of sucrose

[5] For these and other data subjected to the analysis of variance, the partitioning of the degrees of freedom and the mean squares obtained are given in Table 8.

TABLE 2

Effect of granule size on maximum viscosity of starch pastes. Four concentrations of citric acid. Averages for four sources of starch and four concentrations of sucrose

In Brabender units

Normality of citric acid solutions	Granule-sized fraction		
	Small	Medium	Large
	BU	*BU*	*BU*
0	165	184	197
0.050	282	284	305
0.100	243	261	274
0.200	228	236	262
Average	230	241	259

TABLE 3

Effect of granule size on maximum viscosity of starch pastes. Four concentrations of sucrose. Averages for four sources of starch and four concentrations of citric acid

In Brabender units

Concentration of sucrose	Granule-sized fraction		
	Small	Medium	Large
g./100 ml. water or citric acid solution	*BU*	*BU*	*BU*
0	203	198	204
10	236	241	262
20	251	265	293
40	229	260	278
Average	230	241	259

(Figure 2A, B). With the third increment of sucrose, maximum viscosity became lower in the absence of citric acid (Figure 2A)) and changed little in the presence of citric acid, even in the highest concentration used (Figure 2B). This indication of an interaction between citric acid and sucrose may be seen also in Table 4. The drastic thinning effect of citric acid may be seen by a comparison of the terminal viscosities in Figure 2B with those in 2A. The slight thickening effect of sucrose may be seen by a comparison of the terminal viscosities in Figure 2D with those in 2C.

Higher values for maximum and terminal viscosity were obtained for pastes of starch from soft wheat flour than for pastes of starch from hard wheat flour. Higher values also were obtained for pastes of bleached soft wheat starch than for the same starch unbleached. The limited evidence does not permit generalization concerning differences between soft wheat starch and hard wheat starch or the effect of bleach on viscosity of pastes.

Size distributions of pasted granules. Granule-size distribution data cannot be subjected to statistical analysis unless mean values are used, and mean diameters do not adequately characterize starch samples unless the sizes of

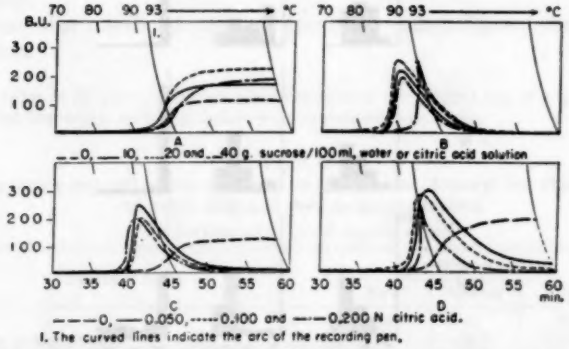

Figure 2. Amylograms illustrating the effect of citric acid and sucrose on viscosity-temperature curves of pastes of unfractionated starch from soft wheat unbleached flour. (A) No citric acid, four concentrations of sucrose. (B) 0.200 N citric acid solution, four concentrations of sucrose. (C) Four concentrations of citric acid, no sucrose. (D) Four concentrations of citric acid, 40 g. sucrose/100 ml. water or citric acid solution.

TABLE 4

Effect of citric acid and sucrose on maximum viscosity of starch pastes. Averages for four sources of starch

In Brabender units

Concentration of sucrose	Normality of citric acid				Average, all conc. of acid
	0	0.050	0.100	0.200	
g./100 ml. water or citric acid solution	*BU*	*BU*	*BU*	*BU*	*BU*
0	132	237	214	201	196
10	189	280	253	235	239
20	220	309	274	254	264
40	155	317	277	260	252
Average	174	286	255	237	

the granules in the samples are unusually uniform. Figure 3 provides a means of making comparisons among granule-size distributions for the pastes of the large-granule fraction of starch from soft wheat unbleached flour. As citric acid concentration was increased, a shift toward a larger number of small granules occurred because of fragmentation of granules. The shift became less pronounced as the concentration of sucrose was increased. The protective effect of sucrose was confirmed by microscopic examination. Both the effect of acid and the effect of sugar were less pronounced on small-granule fractions than on medium- and large-granule fractions.

Iodine uptake by soluble material in pastes. Iodine uptake was not found to be related to granule size. Citric acid (Table 5) increased the iodine uptake, the effect being nearly as great with 0.050 N acid as with 0.200 N acid.

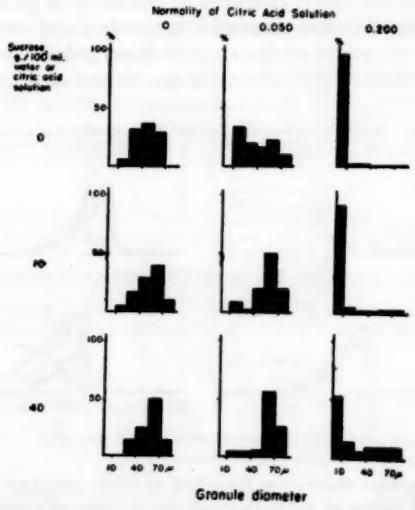

Figure 3. Histograms illustrating the effect of citric acid and sucrose on granule size-frequency distributions in pastes of the large-granule fraction of starch from soft wheat unbleached flour.

TABLE 5

Effect of citric acid and sucrose on uptake of iodine by soluble material in pastes. Averages for four sources of starch. Values are mg. iodine per gram dry starch

Concentration of sucrose	Normality of citric acid			Average, 3 conc. acid
	0	0.050	0.200	
g./100 ml. water or citric acid solution	*mg.*	*mg.*	*mg.*	*mg.*
0	31.5	34.4	34.0	33.3
10	29.7	33.1	34.7	32.5
40	13.0	34.0	33.4	26.8
Average	24.7	33.8	34.0	

Increasing the concentration of sucrose (Table 5) decreased the iodine uptake in the absence of citric acid and had no effect in the presence of citric acid.

Sag of gels. The gels obtained with the small-granule fractions were weaker than the gels obtained with the medium- and large-granule fractions (Table 6).

Most of the pastes prepared with 0.200 N citric acid or containing 40 g. sucrose per 100 ml. liquid failed to form gels. Twelve of those prepared with the next highest concentration of citric acid or sucrose failed to form measurable gels. An arbitrary value of 60 percent sag [b] was supplied for each of these twelve pastes in order to simplify the analysis of the data. Percent sag (Table 7) became progressively greater with increased concentration of citric acid. Percent sag also became progressively greater with increased concentration of sucrose.

DISCUSSION

Relationship between amylose in solution and gel formation. If the amount of iodine taken up by a solution of starch is assumed to represent the amount of amylose in the solution, the following statements may be made concerning the findings of this study:

Citric acid interfered with gel formation and increased the amylose in solution.

Sucrose also interfered with gel formation but decreased the amylose in solution.

[b] The value of 60 was considered representative of the percent sag of a gel which could be unmolded but which collapsed before a measurement was made.

TABLE 6

Effect of granule size and source of starch on sag of gels. Average for nine combinations of citric acid and sucrose concentrations

In percent of original height of gel

Source of starch	Granule-sized fraction			Average, 3 fractions
	Small	Medium	Large	
	%	%	%	%
Soft wheat unbleached	34.7	28.7	28.1	30.5
Soft wheat bleached	31.7	26.1	22.2	26.7
Hard wheat unbleached	31.2	25.8	28.1	28.3
Hard wheat bleached	34.3	26.8	28.8	30.0
Average	33.0	26.8	26.8	

TABLE 7

Effect of citric acid and sucrose on sag of gels. Average for four sources of starch

In percent of original height of gel

Concentration of sucrose	Normality of citric acid			Average, 3 conc. of acid
	0	0.050	0.100	
g./100 ml. water or citric acid solution	%	%	%	%
0	23.2	36.2	43.9	34.4
10	23.6	38.3	53.9	38.6
20	34.0	42.5	55.4	43.9
Average	26.9	39.0	51.1	

The above two statements, when considered together, seem to refute a direct relationship between the amount of amylose in solution and gel strength. However, the following statements also may be made:

Citric acid produced fragmentation of starch granules.

Sucrose protected starch granules from fragmentation.

The results suggest the requirement of at least two factors for gel formation: 1) a sufficient concentration of relatively intact granules to provide a minimum amount of rigidity, 2) enough amylose in solution to serve as "binding" between the granules. Failure of a paste containing a high concentration of sucrose to form a gel might be explained by insufficient binding material in the presence of sufficient "building blocks." Failure of a paste containing a high concentration of citric acid to form a gel might be explained by insufficient "building blocks" in the presence of sufficient binding material. The relatively low strength of gels obtained with the small-granule fractions apparently was due to the small size of the "building blocks," since no difference was found in iodine uptake for different granule-sized fractions.

TABLE 8

Mean squares of measurements

Source of variance	d.f.	Maximum viscosity	Viscosity at 93° C.	Terminal viscosity	Percent sag	Iodine uptake
Source of starch	3	165,893**	80,679**	9,791**	58.41	64.0
Fraction	3	17,035*	9,537*	2,260**	320.30*	31.4[1]
Source x fraction	9	3,166	1,815	292	53.65	31.3[3]
Citric acid	3	141,938**	334,432**	379,428**	3,936.76**[1]	1,014.5**[1]
Sucrose	3	56,577**	59,249**	15,474**	613.77**[1]	451.8**[1]
Acid x sucrose	9	4,240**	24,333**	10,958**	82.62[3]	402.3**[3]
Source x acid	9	3,957**	6,014**	16,574**	65.59[3]	14.6[3]
Source x sucrose	9	1,260*	3,402**	672	60.49[3]	11.3[3]
Fraction x acid	9	394	1,406	1,667*	58.97[3]	59.4[3]
Fraction x sucrose	9	1,438**	3,151*	408	20.31[3]	28.1[3]
Replications	3	602	2,169	73	30.28	597.0**
Residual	186	543	1,270	845	85.89[4]	26.0[5]

[1] 2 d.f.
[3] 6 d.f.
[3] 4 d.f.
[4] 81 d.f. 12 values supplied.
[5] 65 d.f.
* $0.05 \geqq P \geqq 0.01$.
** $0.01 \geqq P$.

No evidence was sought or obtained to indicate whether, for gel formation, the binding material must include the branched component of starch in addition to amylose.

SUMMARY

A factorial experiment was carried out for the purpose of studying the relationship between amylose in solution and gel formation and the effect of granule size, citric acid and sucrose on some properties of pastes and gels of wheat starch.

Amylose in solution increased with increasing concentration of citric acid and decreased with increasing concentration of sucrose. Citric acid produced fragmentation of starch granules, whereas sucrose tended to protect starch granules from fragmentation. The requirement of at least two factors for gel formation is suggested: 1) a sufficient concentration of intact, swollen granules to serve as "building blocks," 2) enough amylose in solution to serve, possibly with some amylopectin, as "binding" between the granules.

Pastes of large-granule fractions thickened at lower temperatures than did pastes of small-granule fractions. Citric acid lowered temperatures of thickening. Sucrose delayed thickening.

The effect of increasing granule size in the absence of citric acid and sucrose was a decrease in maximum and terminal viscosity. In the presence of both citric acid and sucrose, the effect of granule size was reversed, the reversal being due to an interaction between granule size and sucrose.

Maximum viscosity of pastes became higher with the first increment of citric acid and decreased with further increments of citric acid, regardless of the concentration of sucrose. The effect of sucrose was dependent upon the concentration of citric acid.

Weaker gels were obtained with small-granule fractions than with fractions of larger granules. Sag of gels became progressively greater as the concentration of citric acid was increased and as the concentration of sucrose was increased. The highest concentration of either citric acid or sucrose prevented the formation of measurable gels.

Because of the existence of interactions between the factors studied, general statements concerning the effects of a single factor on the properties of starch pastes and gels can be made only when conditions are defined.

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YEASTS OCCURRING IN DROSOPHILA FLIES AND IN FERMENTING TOMATO FRUITS IN NORTHERN CALIFORNIA

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(Manuscript received January 28, 1957)

The vinegar fly, in particular the species *Drosophila melanogaster,* is considered to be one of the serious insect pests in tomato fields, in boxed fruit, and in processing plants during the middle and late harvesting season. The flies deposit numerous eggs in cracks and other damaged portions of the fruit; this is followed soon by the development of larvae, sometimes within one hour after egg deposition *(8)*. As a result, various products made from such tomatoes may attain an insect fragment count exceeding the tolerance set by the Food and Drug Administration. In addition, vinegar fly infestation greatly contributes to the microbial contamination and, consequently, to the fermentative spoilage of tomatoes.

In addition to the studies of Michelbacher and Middlekauff *(8)* in California, a report by Bickley and Ditman *(2)* indicates that the Drosophila problem is equally serious in Maryland and Pepper *et al. (11)* report the same situation in New Jersey. Principal control measures advocated in the publications just cited relate to the application of various insecticides to the fields or to the boxed tomatoes. A recent report by Doyle, Kimball and Olson *(6)* of the National Canners Association indicates that pyrethrin dust applied to stacked boxes in the field or in cannery sheds offers excellent protection against fly infestation.

Intensification of interest in the food habits and nutritional preferences of wild species of Drosophila by Dobzhansky and collaborators has led to several ecological surveys of yeasts found in the alimentary canal of a number of wild species of Drosophila *(12, 14)*. In addition, the yeast flora of the breeding sites of a few wild species has also been studied *(3)*. Certain findings and conclusions of this and related work having a bearing on the present research, may be summarized as follows:

(a) Drosophila flies require yeasts or other microorganisms in their diet for optimal development *(4)*. (b) If given a choice of food containing pure cultures of different yeasts, preferences are clearly demonstrated. Such preferences are not the same for different species of Drosophila *(4, 5)*. (c) Wild species of Drosophila, so far as is known, seem to oviposit in substrates suitable for larval development, which are different from feeding substrates for adults *(3)*. (d) A taxonomic study of the microbial crop contents of Drosophila and of the environmental yeast flora might furnish correlative clues to the natural food sources of the flies *(3, 12)*. (e) With respect to tomatoes, it has been shown that flies deposit eggs readily on the moist surface of sliced sound fruit. However, based on the number of visits to bait cups, it was also shown that macerated tomato fermented by bakers

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yeast exhibited a much greater attractivity for feeding than plain tomato pulp *(8)*. Egg deposition in fermented fruit was not reported in the publication cited.

The main purpose of the present work was to study the food intake of domestic vinegar flies occurring in tomato fields with special emphasis on the yeasts present in their crops. Secondly, a more limited number of samples of fermenting tomatoes were investigated similarly with respect to the yeasts responsible for fermentation in the field. Since the literature does not contain any systematic studies regarding the species of yeast responsible for the fermentation of tomatoes or of the yeasts occurring in the intestinal tract of Drosophila flies caught in such fields, it was considered essential, as an initial step, to study the taxonomy of the yeasts isolated. It was hoped that a comparison of the florae in the flies and in the tomatoes might clarify the feeding habits of a domestic species of Drosophila.

EXPERIMENTAL TECHNIQUE

Collections were made in 4 fields located as follows: Fields No. 1 and 2 on opposite sides of Davis; field No. 3 near Woodland; and field No. 4 near Winters, California. Samples were collected between September 25 and October 25, 1955. Flies were induced to emerge from the tomato plants by shaking the bushes. They were then caught in a net from which they were transferred to a sterile vial. Fermenting tomatoes were picked at the same time and placed in sterile jars. Samples were taken to the laboratory without delay and flies dissected immediately to prevent digestion of the crop content *(13)*. Dissection was done by the procedure described by Phaff *et al. (12)*.

The yeast content of the crops of the flies was studied, rather than streaking whole flies on the plate, in order to obtain a picture of the yeasts ingested during feeding and excluding in this way accidental contaminants adhering to exterior parts of the body. Dissected crops, as well as direct samples from fermenting tomato flesh, were streaked on plates containing 5° Balling malt extract. Since over 98% of the flies caught belonged to *Drosophila melanogaster*, only individuals of this species were studied.

Yeast colonies which appeared on a plate were inspected carefully with a wide-field microscope and those which showed differences in colony morphology were isolated. If later, more than one isolate from the same plate (and therefore from the same fly or tomato fruit) proved to be identical species, they are reported here as single isolations.

Yeasts were identified by the system of Lodder and Kreger van Rij *(7)* except that the number of carbon compounds used to determine their assimilation characteristics was increased by adding compounds similar to those recommended by Wickerham *(16)*. Assimilations were determined by the replica plating tcehnique described by Shifrine *et al. (15)*. A result of this approach was that certain of our most common species placed into synonomy with related ones by Lodder and Kreger van Rij, were reestablished as valid species. Detailed taxonomic considerations of the isolates will be published elsewhere.

RESULTS AND DISCUSSION

Yeasts isolated are listed in Table 1. A total of 181 cultures was obtained. One hundred and sixty-two of these were from flies and 19 from tomatoes. This is due to the fact that many more flies were studied than tomato fruits. The main purpose of the study was to compare the diet of *D. melanogaster* with that of certain wild species studied earlier *(12, 14)*. Only rarely were flies devoid of yeasts in their crop and usually the number of colonies per crop varied from several dozen to several hundred. Since Drosophila flies are known to digest yeast cells rapidly *(13)*, the number of yeast cells per crop undoubtedly depends in part on the time lapse since the last feeding of the fly. Bacteria, although present on many of the plates, were not isolated

in the present study. Since all the major species of yeast occurred with approximately equal frequency in the 4 areas studied, separate figures for the different fields will not be reported.

Several interesting conclusions can be drawn from Table 1. Although only a limited number of tomato samples were plated, the results show that all major species of yeast occurred both in the intestinal tract of the flies and in the fermenting fruit. This was demonstrated not only by the reported isolations (see Table 1), but also by the fact that between 90 and 100% of

TABLE 1

Yeasts isolated and sources of isolation. The numbers represent the combined isolates from the four fields

Name of isolates	Number of isolates identified	
	from Drosophila	from fermenting tomatoes
Hanseniaspora uvarum (Niehaus) Shehata *et al.*	78	9
Kloeckera apiculata (Reess emend. Kloecker) Janke	12	1
Pichia kluyveri Bedford	61	7
Candida krusei (Cast.) Berkhout	8	---
Candida mycoderma (Reess) Lodder et Kreger-van Rij	1	---
Candida parapsilosis (Ashf.) Lang. et Talice var. *intermedia* Kreger-van Rij et Verona	1	---
Candida catenulata Diddens et Lodder	1	---
Non-classified pink yeast	1	1
	163	18

the colonies appearing on a plate from either tomatoes or flies were apiculate yeasts *(Hanseniaspora* and *Kloeckera)* and *Pichia* in approximately equal numbers. The other isolates were present in small numbers as judged by colony morphology. This demonstrates rather conclusively that the major food source for the adult flies consists of the moist tissues of fermenting tomatoes. Since Michelbacher and Middlekauff *(8)* have shown that flies oviposit heavily on freshly sliced (or damaged) sound tomatoes, it appears then that even in tomato fields the feeding substrate (fermenting fruits) and breeding substrate (damaged but sound tomatoes) are different. Naturally during egg deposition and larval development, the substrate is apt to become contaminated and active microbial fermentation takes place. Due to the proximity of feeding and breeding tissue (both may be part of the same tomato fruit) it would appear that for domestic species, the larval and adult food supply is identical, although it may be in different stages of decay. This is in contrast to the situation for certain of the wild species *(3)*. In their search for new breeding places, the flies are likely to be an important vector in the spread of microbial spoilage in the field.

Table 1 also shows that the yeast flora involved is highly specific and that essentially the flora in the 4 areas consists of only 2 species, *Hanseniaspora uvarum* (including its imperfect form *Kloeckera apiculata*) and *Pichia kluyveri*. Two of the cultures termed *Candida krusei* represent the imperfect form of *P. kluyveri*, as judged by their carbon assimilation patterns. The other six, although identical to each other, had a different carbon assimilation

pattern, but by the system of Lodder and Kreger van Rij *(7)* they fall in *C. krusei*. The other species listed occurred very infrequently and only a few colonies of these appeared on the plates. They are probably of minor importance. Some brief comments regarding the nomenclature of the 2 major species are in order. *Hanseniaspora uvarum* was originally named *Kloeckeraspora uvarum* by Niehaus *(10)*. It is an apiculate yeast, which forms one or rarely two spherical ascospores per ascus. Shehata *et al. (11)* transferred this organism to the genus *Hanseniaspora* on the basis of its close relationship to this genus. *K. apiculata* is physiologically and morphologically identical to *H. uvarum*, except for its inability to produce ascospores. A single isolate of *Pichia kluyveri* was described and named by Bedford in 1942 *(1)*. Lodder and Kreger van Rij *(7)* placed it in synonomy with *P. fermentans* to which it is obviously related. However, we shall retain it as a valid species, since the type species of *P. kluyveri* and all 68 isolates collected were unable to utilize the pentose sugar D-xylose, whereas *P. fermentans* grows well on this sugar as a single carbon source. Moreover, *P. fermentans* forms a well-developed pseudomycelium, while *P. kluyveri* lacks pseudomycelium forming ability. It is curious that no species of the genus *Saccharomyces* were isolated, since these strongly fermentative yeasts have long been assumed to be prominent among the spoilage yeasts of fruits. Another yeast that might have been expected in fermenting tomatoes on the basis of its common occurrence in fermenting fruit is *Hanseniaspora valbyensis*. This yeast is morphologically and physiologically identical to *H. uvarum* except that it produces 4 small hat-shaped spores instead of one or two spherical spores. No isolates of this yeast were found, however, in spite of a careful search of the isolation plates. Since this yeast sporulates readily on malt agar, its presence is readily detected on isolation media. Both major species ferment glucose, fructose and mannose vigorously, but no other sugars. The sugar content of tomatoes is about 5% and consists mostly of glucose and fructose *(17)*. Thus, *H. uvarum* and *P. kluyveri* may be considered the principal fermentative spoilage yeasts in the important tomato producing areas studied.

As to the possible source of the spoilage yeasts, the following evidence points to the flies as the vector in contamination. Yeasts have been reported in the tissues of certain sound fruits *(9)*. If such yeasts would exhibit growth and fermentation after the fruit reaches a soft ripe maturity, it might explain fermentative spoilage. However, samples obtained aseptically from the flesh of soft ripe fruit in 1955 as well as in 1956 never yielded any growth.

Although it is known that the apiculate yeasts are commonly found in soil samples *(7)*, we were unable to detect the presence of these yeasts or of *P. kluyveri* in the dust (or the surface washings) of sound tomatoes.

Attractivity experiments have shown that apiculate yeasts are among the most highly attractive yeasts for a number of species of Drosophila *(4, 5)*. Since these yeasts occur on all types of deciduous fruits, it may be postulated with reasonable certainty that the flies migrated from peach and prune orchards, vineyards, and similar places when the food supply for Drosophila flies was exhausted after completion of the harvest. Since tomato fields constitute the last major reservoir of food for the vinegar fly before the arrival of cold weather, the large population growth can be understood readily. Yeast contamination on the exterior body parts would form a sufficient

inoculum during ovipositing in damaged sound tomatoes to start the cycle in tomato fields. The absence of *S. cerevisiae* makes it unlikely that flies migrated from pomace piles at nearby wineries. Although vinegar flies may justifiably be suspected as the major vectors for the spread of yeasts, the participation of other insects is not necessarily excluded.

A comparison of yeasts associated with *D. melanogaster* and yeasts isolated from non-domestic species of *Drosophila (12, 14)* shows that *H. uvarum* and *K. apiculata* were isolated from both sources. On the other hand, *P. kluyveri* was not found in the wild species and the latter group contained many species of *Saccharomyces, Hansenula* and *Torulopsis* which were absent in the flies from tomatoes. It is quite possible that the introduction of a mixed population of yeast in tomatoes by migrating Drosophila starts a competitive pattern of growth, with the apiculates and *P. kluyveri* gaining the upper hand when the spoilage is well underway. This flora in turn characterizes the predominant yeasts in the crops of *D. melanogaster.*

SUMMARY

A study has been made of the yeasts occurring in the alimentary canal of *Drosophila melanogaster* occurring in four tomato fields in northern California during the 1955 season. For comparison, a smaller number of samples of fermenting tomatoes were also included. Yeast flora of the flies and the tomatoes, collected in four widely separated fields, was essentially identical. Of the 181 yeasts isolated, 55.2% represented *Hanseniaspora uvarum* and its imperfect form *Kloeckera apiculata* (87 and 13 isolates, respectively). *Pichia kluyveri* formed 38.7% of the isolated yeasts (68 isolates and 2 imperfects of this species placed in *C. krusei*). Population wise, the two main species each represented between 40 and 50% of the total yeast population.

This highly specific yeast flora varies considerably from the yeast flora in wild species of Drosphila, although some common features exist. It has been shown that the flies feed on fermenting tomatoes. Since ovipositing is done primarily on cracked or damaged sound fruit, the vinegar fly can be considered an important vector in spreading microbial spoilage in tomato fields. Some evidence is also presented that introduction of the spoilage yeasts in tomato fields is the result of flies migrating from nearby deciduous fruit orchards and similar habitats.

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THE EFFECT OF VARIOUS MUST TREATMENTS ON THE COLOR AND TANNIN CONTENT OF RED GRAPE JUICES

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(Manuscript received February 8, 1957)

Colored pigments of grapes, with the exception of a few varieties with colored pulp, are found entirely in the epidermal cells of the skins. Tannin occurs chiefly in the cells of the skins, seeds and stems, with very little being found in the pulp.

Cells consist of: the cell wall; cytoplasm, which lines the inner surface of the cell wall; plastids, which are contained in the cytoplasm; and a vacuole, which is completely surrounded by the cytoplasm. Some pigments are contained in the plastids; however, most of the pigments and some of the tannins are found in the vacuole.

The cell wall is permeable, but the cytoplasmic membrane is semipermeable and thus restricts the passage of pigments from the plastids and pigments and tannins from the vacuole. These two components cannot pass out of the cell unless the semipermeability of the membrane is lost, due to the death or injury of the cell.

Primary factors affecting the semipermeability of the cell are thought to be: temperature of the must, time of contact of juice with the pomace, alcohol content, sulfur dioxide content, and carbon dioxide content of the solution. Agitation is also thought to affect color and tannin release by mechanical injury of the cells.

A number of previous studies have been made on color and tannin extraction from California grapes. Amerine *(2)* found that extraction increased with increasing temperature, and also with increased agitation of the must. Berg and Akiyoshi *(6)*, in seven commercial fermentations, found that the color content of the juice reached a maximum at various points during the fermentation while tannin content increased as long as the juice remained in contact with the pomace. They were unable to predict reliably the point of maximum color extraction from either the fermentation time or the alcohol content of the juice.

The effect of sulfur dioxide has been discussed by Amerine and Joslyn *(4)*. They reported that addition of 250 p.p.m. of sulfur dioxide prior to fermentation resulted in a wine with approximately 50% more color (as determined by color standard comparison) than wine from must fermented with 50 p.p.m. of sulfur dioxide.

Amerine and De Mattei *(3)* found that confining whole grapes in a closed jar resulted in an increase in the percentage of carbon dioxide in the container and a greater release of color when the grapes were crushed. Whether the increase in color was due to anaerobic respiration or to asphyxiation due to the increased concentration of carbon dioxide in the container, or to a combination of the two could not be determined from this experiment. In another experiment they replaced the air in the jars with oxygen, carbon dioxide and nitrogen. An increase in color over the closed check was obtained with all the gases, with carbon dioxide having the least effect and nitrogen the most.

Although the percentage-wise differences from the control were appreciable, the total amount of color obtained was small.

EXPERIMENTAL

Grapes used in these studies were grown in the University vineyard at Davis.

Must treatment. Experiments in which carbon dioxide and nitrogen gas were added were conducted with 25-gallon lots in the controlled fermentation equipment previously described by Amerine *(1)*. All other experiments were with 5-gallon lots in 20-gallon open earthenware crocks. Unless otherwise noted each lot was punched twice daily. All fermentations were at comparable temperatures in the range of 18°–24° C.

Additions of sulfur dioxide or alcohol were made immediately after crushing, unless otherwise noted. Where fermentation was desired a 1% culture of Montrachet yeast strain was added 4 hours later. Where fermentation was not desired 40 p.p.m. of Captan (N-trichloromethylmercapto-4-*cyclo*hexene-1,2-dicarboximide) was added immediately after crushing. Captan, which effectively prevented the action of both yeast and bacteria, did not have an appreciable effect on either the color or tannin contents of the juice.

Sampling. One-pint samples of juice were drawn periodically from the 25-gallon lots. In the case of the 5-gallon lots it was felt that periodic sampling would appreciably change the juice to pomace ratio. Therefore, sufficient individual 5-gallon lots were prepared to give the desired number of samples and each lot used as one sample. Uniformity of the lots was ensured by crushing into a 50-gallon drum, stirring the contents, and then ladling, while stirring, one quart amounts of must into each crock in turn until 20 additions had been made.

One-pint samples of the screened 5-gallon lots were removed for analysis. The remainder of each lot was kept for further treatment and analysis.

Analyses. The samples of juice were filtered immediately through diatomaceous earth and then analyzed. The degree Brix, pH, total acidity, total sulfur dioxide and tannin content (Folin-Denis) were determined by methods given in the section on wines in the A. O. A. C. *(5)*. The alcohol was determined by distilling 100 ml. of sample and calculating the percent alcohol from the boiling point as determined in an ebulliometer.

Color was determined by making transmission measurements on the Coleman Model II spectrophotometer. The percent transmission was measured at 25 mμ intervals over the range of 400 to 700 mμ, using a 2 mm. cuvette with water as the reference liquid. From these measurements the dominant wavelength, percent purity and percent brightness (% Y) were determined, using the weighted ordinate method of Hardy *(10)*.

The color difference, Δ C, was calculated from the trichromatic coefficients and brightness values. The Δ C value as determined from the nomographs based on Davidson's *(9)* calculations is expressed in McAdam units. A McAdam unit is proportional to the minimum perceptible difference between two colors as judged by the average observer.

Transmission measurements were made on each sample before and after the addition of 0.04 ml. of 3% hydrogen peroxide to 10 ml. of wine. A reaction time of 10 minutes was required to complete the splitting of the colorless sulfur dioxide—pigment complex causing the pigment to reappear in the colored form. It was found that addition of the hydrogen peroxide had no effect on fermented juices which had received an addition of 100 p.p.m. of sulfur dioxide before fermentation. However, non-fermented juices with 100 p.p.m. showed an appreciable color increase as did fermented juices which had been sulfited with 250 p.p.m. of sulfur dioxide. Non-sulfited juices showed a slight decrease in color. Since the object of this study was to determine the actual effect of various must treatments on color, the color data reported here is that without the masking effect of sulfur dioxide.

RESULTS AND DISCUSSION

Ten experiments were run in an attempt to determine the effect of carbon dioxide, fermentation, sulfur dioxide, alcohol and time on the color and tannin contents of the juices. Tables 1–10 give the must analyses, must treatments, sampling times, alcohol and tannin contents of the juices, % Y of the juices and △ C between samples.

As the must treatments failed to show an appreciable effect on pH and total acidity these values are not included in the tables. The total sulfur dioxide content is also omitted, as no consistent relationship of amount remaining in the juices to must treatment was established. Of the color characteristics only % Y (brightness) is given, as it was felt that it is the best single expression of color density. A decrease in % Y indicates an increase in color density. The color difference, $\triangle$ C, is also given because it includes not only brightness but also purity of color and hue.

Table 1 gives the must analysis of the various lots of grapes used in the experiments. Where more than one number appears in the same line in the column headed "Experiment No." it indicates that these experiments were run on the same lot of grapes.

Effect of carbon dioxide. Data in Experiment 1, Table 2, compare the effect of added carbon dioxide with that of added nitrogen during fermentation. The quantity of gas added was approximately equal in volume to that produced by the complete fermentaiton of this amount of must. Though samples taken from the nitrogen treated must averaged slightly higher in both color density and tannin content the difference was not statistically significant.

TABLE 1

Must analysis of the lots of grapes used

Experiment No.	Grape variety	° Brix	pH	Total acidity g./100 ml. as tartaric
1	Valdepenas	19.2	3.30	1.09
2	Carignane	18.4	3.58	0.81
4, 5	Carignane	18.7	3.55	0.94
6, 8	Zinfandel	21.8	3.66	0.81
7	Carignane	18.0	3.77	0.68
9, 10, 11	Carignane	24.6	3.84	0.60

TABLE 2

Comparison of the effect of addition of carbon dioxide with that of nitrogen

Time sampled in hours	% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		$\triangle$ C
	Experiment 1[1]						
	CF 108	CF 109	CF 108	CF 109	CF 108	CF 109	108:109
10	0.3	0.4	0.068	0.058	66.0	65.7	0.6
18	1.0	1.1	0.078	0.084	58.6	56.3	3.5
34	5.9	6.1	0.098	0.100	45.9	48.8	5.5
42	7.5	7.5	0.124	0.128	43.6	44.2	1.4
58	9.8	9.9	0.118	0.136	43.7	44.2	0.3
64	10.0	9.7	0.114	0.124	46.5	41.0	8.0
81	9.7	9.7	0.130	0.124	41.3	40.5	2.0
	Experiment 2[2]						
	CF 128	CF 129	CF 128	CF 129	CF 128	CF 129	128:129
97	0.0	0.0	0.076	0.076	58.8	57.8	1.4

[1] Fermented, 100 p.p.m. of sulfur dioxide added, 1 liter per minute of carbon dioxide added to CF 108 and 1 liter per minute of nitrogen to CF 109.

[2] Not fermented, cap not punched, 100 p.p.m. of sulfur dioxide added, 1 liter per minute of carbon dioxide added to CF 128 and 1 liter per minute of nitrogen to CF 129.

Experiment 2 was run as it was thought that the carbon dioxide evolved by the fermentation might have obscured the effect of the added gases. The same results were obtained as in Experiment 1.

Comparisons in Table 2 failed to show any significant differences between the effects of the two gases. Differences would have been indicative of a permeability effect on the cells. However, data in Table 3 show a different picture. Samples from the nitrogen treated must were significantly greater in color density and tannin content than those from the lot to which no gas had been added. Samples from the carbon dioxide treated must (compare Table 2 with Table 3) were also higher in color density and tannin content, but the difference in this case was not statistically significant.

It is known that agitation has an effect on color and tannin extraction. Thus, the increased tannin and color obtained by adding gas could be due entirely to the resulting increase in agitation. The data of Tables 2 and 3

TABLE 3

Effect of addition of nitrogen during fermentation

Time sampled in hours	% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		ΔC
	Experiment 1[1]						
	CF 109	CF 110	CF 109	CF 110	CF 109	CF 110	109:110
10	0.4	0.4	0.058	0.062	65.7	68.4	3.0
18	1.1	0.9	0.084	0.068	56.3	60.3	6.6
34	6.1	5.9	0.100	0.088	48.8	58.6	14.1
42	7.5	8.7	0.128	0.104	44.2	51.2	11.0
58	9.9	9.7	0.136	0.134	44.2	41.8	3.5
64	9.7	9.8	0.124	0.118	41.0	45.7	9.7
81	9.7	9.9	0.124	0.104	40.5	45.9	10.1

[1] 100 p.p.m. of sulfur dioxide added, 1 liter per minute of nitrogen added to CF 109, no gas added to CF 110.

do not support this explanation as equal quantities of the two gases were introduced during fermentation. Evidently, both gases increase the permeability of the cells with nitrogen having the greater effect of the two. This finding confirms that of Amerine and De Mattei *(3)*. It also agrees with their opinion that the effect is not great.

Effect of sulfur dioxide. In Experiment 4, Table 4, though the color content of the sulfited juice reached a maximum early in the fermentation while the non-sulfited juice continued to increase in color to the end of the fermentation, the sulfited juice at all stages of the fermentation had the greater color and tannin content. In Experiment 10 both increased in color to the end of the fermentation with the sulfited juice again having the greater color and tannin content. This confirms the findings of Bioletti and Cruess *(8)* who reported an average color increase of 23% in wines from sulfited musts. Where no fermentation occurred, as in Experiment 9, Table 5, where the musts were fortified, addition of sulfur dioxide increased the tannin and color contents of the juice.

In Experiment 8, Table 6, where two musts were fermented with respectively 100 and 250 p.p.m. of added sulfur dioxide, the results obtained are

TABLE 4

Effect of sulfur dioxide on fermenting musts

Time sampled in hours		% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		ΔC
Experiment 4[1]								
7950A	7950B	7950A	7950B	7950A	7950B	7950A	7950B	A:B
20	8	0.1	0.1	0.056	0.032	74.5	90.5	19.1
35	32	1.6	1.7	0.055	0.048	71.8	79.6	10.1
42	37	2.4	2.7	0.063	0.049	72.8	79.5	9.1
49	44	4.1	4.0	0.064	0.057	74.8	78.5	5.7
53	48	5.0	4.5	0.068	0.055	72.4	79.5	10.4
57	53	5.8	5.9	0.073	0.061	70.1	77.0	9.4
59	57	6.8	6.5	0.065	0.061	71.0	75.4	6.4
60	59	7.2	7.3	0.069	0.059	71.4	74.5	5.3
66	64	8.6	8.7	0.069	0.059	69.6	74.7	6.6
115	115	10.3	10.4	0.063	0.056	71.5	73.5	2.8
Experiment 10[2]								
		8148A	8148B	8148A	8148B	8148A	8148B	A:B
	27	0.2	0.3	0.081	0.076	67.9	75.5	7.0
	75	10.1	10.0	0.119	0.108	49.5	52.2	5.1
	123	14.6	14.4	0.120	0.112	48.1	50.2	4.0

[1] 100 p.p.m. sulfur dioxide added to 7950A, no sulfur dioxide added to 7950B.
[2] 100 p.p.m. sulfur dioxide added to 8148A, no sulfur dioxide added to 8148B.

TABLE 5

Effect of sulfur dioxide on non-fermented, fortified musts

Time sampled in hours	% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		ΔC
Experiment 9[1]							
	8146A	8146B	8146A	8146B	8146A	8146B	A:B
123	14.2	14.5	0.180	0.166	34.8	39.5	12.1

[1] 8146A fortified to 14.2% alcohol and 100 p.p.m. sulfur dioxide added. 8146B fortified to 14.5% alcohol, no sulfur dioxide added.

interesting. The juice of the 100 p.p.m. sulfur dioxide must attained maximum tannin and color content at 5.4% alcohol with no appreciable change thereafter. The juice of the 250 p.p.m. sulfur dioxide must attained maximum color at 0.2% alcohol, and then showed a progressive decrease in color to the end of the fermentation. The tannin content showed a progressive increase throughout the fermentation. The juice of the 250 p.p.m. sulfur dioxide must had the greater tannin content throughout the fermentation, and the greater color up to 7.9% alcohol.. At the end of the fermentation the color content of the two juices was practically the same. From this experiment it appears that part of the color initially released in the juice of the more heavily sulfited must is unstable, and progressively disappears with continued fermentation. This finding does not agree with that of Amerine and Joslyn *(4)* who reported approximately 50% greater color in the juice of the more heavily sulfited must at the conclusion of fermentation.

Effect of fermentation. The object of these experiments was to determine the contribution of fermentation to the color and tannin content of the

TABLE 6

Comparison of effect of 100 p.p.m. sulfur dioxide with that of 250 p.p.m. on fermenting musts

Time sampled in hours		% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		ΔC
Experiment 8[1]								
8019A	8019B	8019A	8019B	8019A	8019B	8019A	8019B	A:B
20	29	0.3	0.2	0.082	0.099	68.0	44.9	40.0
59	70	5.4	5.5	0.101	0.112	58.6	51.3	12.5
72	83	8.0	7.9	0.072	0.116	57.8	53.8	7.8
117	141	12.2	12.4	0.099	0.120	59.5	60.2	0.6

[1] 100 p.p.m. of sulfur dioxide added to 8019A and 250 p.p.m. to 8019B.

juice. This contribution involves temperature, time, alcohol and a fourth factor consisting of all other possible effects which are produced by fermentation. If the first three are held constant, the effect of the fourth can be measured.

In Experiment 6 the individual lots of Series 8018A were fortified to the alcohol contents given in Table 7. Corresponding lots of Series 8018B received additions of water equal in volume to the alcohol added to the lots of Series 8018A. The lots of Series 8018B were then allowed to ferment until they had reached the approximate alcohol content of the corresponding lots of Series 8018A, at which time both lots were drawn off the skins. Conditions of the test were approximately satisfied in that the temperature was controlled, the time was the same, and the alcoholic contents were approximately the same at the end of the various time intervals. Complete satisfaction of the conditions of the test would require frequent small additions of alcohol to the

TABLE 7

Effect of fermentation

Time sampled in hours	% Alcohol ml./100 ml.		Tannin g./100 ml.		% Y		ΔC
Experiment 6[1]							
	8018A	8018B	8018A	8018B	8018A	8018B	A:B
20	0.2	0.3	0.081	0.081	70.3	68.1	3.7
31	3.5	4.4	0.110	0.091	57.9	58.9	3.2
34	4.4	5.2	0.124	0.100	57.0	59.5	2.6
38	5.2	6.0	0.120	0.092	54.6	59.9	4.1
43	5.9	7.2	0.110	0.099	53.4	58.3	3.8
47	7.0	7.8	0.120	0.096	56.4	60.1	2.9
56	7.6	8.0	0.128	0.098	51.0	59.8	10.0
60	8.3	9.2	0.124	0.102	54.6	57.4	2.1
Experiments 9, 10[2]							
	8146A	8148A	8146A	8148A	8146A	8148A	A:A
123	14.2	14.6	0.180	0.120	34.8	48.1	18.5

[1] Added 100 p.p.m. of sulfur dioxide to both series. Added alcohol to Series 8018A, not fermented. Added water to Series 8018B, fermented.

[2] Added 100 p.p.m. of sulfur dioxide to both lots. 8146A fortified to 14.2% alcohol, not fermented. 8148A not fortified, fermented to 14.6% alcohol.

non-fermented must to parallel the gradual increase in alcohol content in the fermenting must. However, Experiment 6 should give some measure of the effects of fermentation.

Reference to Table 7 shows that lots which fermented had the least color and tannin content. There are two possible explanations: the fermentation effects cause a measurable loss in tannin and color; or the gradual increase in alcohol to a specified amount in a given time does not effect as much color and tannin release as the full amount of alcohol does in an equal period of time. The data in Experiment 6 favor the second explanation as does that in Experiments 9 and 10. In the latter, even though the fortification resulted in a must dilution of 15%, the juices of the fortitfied musts contained more color and tannin than the juices of the undiluted musts which had been fermented for the same length of time and to the same alcoholic content. Although fermentation effects (other than alcohol and temperature increase) probably contribute to tannin and color release, as is indicated by the data in Table 3, the dominant factors seem to be alcohol and time.

Effect of alcohol. In Experiment 7, Table 8, the effect of alcohol on color and tannin was determined in 3 lots of the same must containing respectively 0, 7.3 and 18.2% alcohol. As the samples were drawn from the 0 and 7.3% alcohol musts, they were fortified to approximately 18.2% alcohol in order to

TABLE 8

Effect of alcohol

Lot No.	Time sampled in hours	% Alcohol ml./100 ml.	Tannin g./100 ml.	% Y	Comparison	Δ C
			Experiment 7[1]			
7951A	121	18.1	0.068	76.0	A : B	5.0
7951B	121	18.6	0.078	72.5	B : C	13.4
7951C	121	18.2	0.103	63.0	A : B	18.7
			Experiments 9, 11[2]			
8146B	123	14.5	0.166	39.5		
8149A	123	15.9	0.231	27.9	B : A	21.2

[1] Not fermented; 100 p.p.m. of sulfur dioxide added. Stored at the following alcohol levels for 121 hours; 7951A at 0.0%, 7951B at 7.3%, 7951C at 18.2%. Fortified to alcohol contents given in table after drawing off.

[2] Not fermented. 8146B stored at 14.5% alcohol. 8149A stored at 47.6% alcohol; the alcohol content was reduced to 15.9% by vacuum distillation after drawing off.

have the same dilution in all samples. As seen in Table 8, there was a progressive increase in both tannin and color content with increasing alcohol concentration.

Experiment 7 raised the question of the maximum color obtainable from a given lot of grapes. Experiments 9 and 11 were designed to answer that question. Two lots of the same must were fortified to 14.5 and 47.6% alcohol, respectively, and then stored at 20° C. for 123 hours. Before analysis the 47.6% alcohol juice was vacuum distilled to reduce the alcoholic content to a level comparable to that of the lower alcohol juice.

The pomace of the high alcohol must was gray at the time of drawing off, indicating almost complete extraction of the color. The pomace from the low

alcohol must had the usual red color. This visual observation was confirmed by the color measurements which showed that a great deal more color had been extracted from the same quantity of grapes by the high alcohol juice. The tannin content was also much greater.

Another lot of the same must fermented to 14.5% alcohol had a % Y of 50.2, the lot fortified to 14.5% alcohol 39.5, and the lot fortified to 47.6% alcohol 27.9. From this we can conclude that the usual fermentation extracts, at the most, not more than two-thirds of the color originally present in the grapes, and that alcohol in contact with the must for the same period of time extracts more color and tannin than does a normal fermentation to the same alcohol level.

Effect of time. Extraction of the pigments from grape skins involves both the loss of the semipermeability of the cells in the skins, and diffusion of the pigments through the cell walls into the surrounding liquid. The percentage of cells losing their semipermeability is dependent upon temperature, agitation, the kind and concentration of substances present which affect the semipermeability of the cells, and the reaction time. The ensuing diffusion of the released pigment into the surrounding juice also requires time.

The experimental conditions in these experiments were designed to test the effect of reaction time on color and tannin extraction. In Experiment 7, Table 9, the juices of the musts containing 0 and 7.3% alcohol reached maximum tannin and color content in the same period of time. Juice of the 18.5% alcohol must required the same length of time to attain maximum tannin content, but a much shorter period to reach maximum color content. In Experiment 9, addition of 100 p.p.m. of sulfur dioxide had no effect on reaction rate. These experiments show that time is an important factor in color and tannin extraction as a minimum reaction time was required to reach equilibrium. They also show that reaction time varies with conditions.

Mechanism of color extraction. It is believed that degree of loss of semipermeability governs not only percentage of total color released, but also determines the diffusion rate. It is also believed that degree of loss of semipermeability required to achieve a certain diffusion rate varies with the must.

In Experiment 7, Table 9, the total color obtained paralleled the alcoholic concentrations of 0.0, 7.3 and 18.5%. In Experiments 9 and 11, Table 8, the total color in the juice was much greater at 47.6% alcohol than at 14.5%. An alcohol affects the semipermeability of the cells these experiments substantiate the widely accepted theory that color release is dependent upon degree of loss of semipermeability.

Experiment 7, Table 9, supports the theory that degree of loss of semipermeability determines the diffusion rate. At the 0.0 and 7.3% alcohol levels the color reached a maximum somewhere between 25 and 73 hours. At the 18.5% alcohol level the color not only reached a maximum in a shorter period (somewhere between 0 and 25 hours), but total amount of color was greater. The work of Berg and Marsh *(7)*, who found no color increase after 30 minutes following heat treatment of the must, also supports this theory. Evidently, if the degree of loss of semipermeability is high, the diffusion of the pigments into the juice is complete in a very short time.

The theory that degree of loss of semipermeability required to achieve a certain diffusion rate varies with the must is supported by the data of Experi-

TABLE 9
Effect of time

Sample No.	Time sampled in hours	% Alcohol ml./100 ml.	Tannin g./100 ml.	% Y	Comparison	Δ C
			Experiment 7[1]			
7951A–1	25	19.1	0.061	80.8		
2	73	20.0	0.072	75.2	1:2	5.9
3	121	18.1	0.068	76.0	2:3	2.5
7951B–1	25	19.3	0.072	75.3		
2	73	19.4	0.083	71.2	1:2	4.4
3	121	18.6	0.078	72.5	2:3	2.6
7951C–1	25	19.0	0.084	64.6		
2	73	18.6	0.105	64.7	1:2	1.4
3	121	18.2	0.103	63.0	2:3	2.8
4	241	17.2	0.104	63.9	3:4	1.6
			Experiment 9[2]			
8146A–1	27	14.5	0.105	50.3		
2	75	14.5	0.176	37.6	1:2	19.6
3	123	14.2	0.180	34.8	2:3	6.4
8146B–1	27	15.0	0.109	56.0		
2	75	14.5	0.160	41.4	1:2	21.7
3	123	14.5	0.166	39.5	2:3	3.3

[1] Not fermented; 100 p.p.m. of sulfur dioxide added. Stored for the indicated times at the following alcohol levels: 7951A at 0.0%, 7951B at 7.3%, 7951C at approximately 18.0%. Fortified to alcohol contents given in the table after drawing off.

[2] Not fermented; stored at approximately 14.5% alcohol for the times indicated in the table. Added 100 p.p.m. of sulfur dioxide to 8146A, none to 8146B.

ments 7 and 9, Table 9. In Experiment 9, the color increased up to 123 hours at an alcohol level of 14.5%, while in Experiment 7, at the lower alcohol level of 7.3%, the color reached a maximum in 73 hours. In these experiments total amount of color found in the various juices at equal diffusion times varied widely. If equal amounts of color required the same diffusion time this would not be true. Therefore, the time required for the released color to diffuse into the juice is not dependent upon total amount of color released but upon characteristics of the must, which determine degree of loss of semipermeability attained under a given set of conditions. Thus, we can conclude that the degree of semipermeability required to achieve a certain diffusion rate varies with the must, or must variation affects the degree of loss of semipermeability attained under a given set of conditions.

Effect of time and alcohol. Berg and Akiyoshi *(6)* reported that neither fermentation time nor alcohol produced could be used to predict the point at which maximum color was attained during fermentation. Because of subsequent work by the writers their previous work was re-examined. This resulted in the data given in Table 10, which was compiled from the fermentations conducted during the 1955 and 1956 vintages. In this table the average color attained at a given alcohol content in 11 fermentations is compared with the average maximum color.

Approximately 80% of the maximum color content was attained at an average alcohol level of 3%. At 6% alcohol the color was found to be almost equal to the maximum, with the average difference 1.3% Y and the greatest

individual difference from maximum color only 4.6% Y. Evidently most of the extraction effect is achieved by the time 3% alcohol has been produced, with only an occasional small additional effect of increased alcohol content beyond 6 percent.

The degree of color extraction from the cells is determined by the concentration of the substances causing the loss of the semipermeability of the cells and the time during which the substances exert their effect. The wide range in elapsed fermentation times at the various alcohol levels indicates that the time required to exert maximum effect is usually much less than the fermentation time. Because of this the alcohol content is the best indicator for determining the time of drawing off of the juice in relation to the desired percentage of the maximum color obtainable by fermentation to dryness on the skins. It should be pointed out, however, that previous work by the writers *(6)* indicated a relationship between length of time on the skins and the color content of the aged wine. If further work should prove that increased time on the skins resulted in greater color retention during aging, this would have to be considered in determining the time of drawing off.

TABLE 10

Effect of alcohol content at time of draw on % Y of the juices

% alcohol at time of draw	Hours		% Y	
	Range	Average	Range	Average
3	25–80	53	17.5–73.5	39.9
6	34–98	84	10.1–70.3	34.2
Maximum[1]	34–122	110	10.2–71.5	32.9

[1] Alcohol content at the conclusion of the fermentation.

SUMMARY

Carbon dioxide had less effect on color and tannin extraction than did nitrogen. Bubbling nitrogen through a fermenting must significantly increased the color and tannin contents of the juice. Addition of 100 p.p.m. of sulfur dioxide to musts increased the color and tannin contents of the juices. With 250 p.p.m. the color extraction is greater up to 8% alcohol, and the same at the end of fermentation. The juice with 250 p.p.m. had the greater tannin content throughout the fermentation.

Fermentation does not extract as much color and tannin as does fortification of the must to the same alcohol levels. Generally, not more than two-thirds of the color originally present in the grapes is extracted during fermentation. Of the total color extracted during fermentation approximately 80% is usually obtained during the production of the first 3% of alcohol, most of the balance during the production of the next 3%, with only an occasional small increase during the remainder of the fermentation. The alcohol content of the must may be used as a guide in determining the percent of the total color extracted during fermentation.

Musts fortified to various alcohol levels show a progressive increase in both the tannin and color contents of the juices with increasing alcohol concentration.

The degree of loss of semipermeability suffered by the cells appears to determine not only the percentage of the total color released but also the

diffusion rate. Must variation affects the degree of loss of semipermeability attained under a given set of conditions. In this study of the various agents which affect the semipermeability of the cell the one of greatest importance in both tannin and color extraction is alcohol.

Acknowledgment

We are indebted to Dr. M. A. Amerine and Mr. C. Ough for conducting the fermentations on the effect of carbon dioxide and nitrogen.

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SOME VOLATILE AROMA CONSTITUENTS OF *VITIS VINIFERA* VAR. MUSCAT OF ALEXANDRIA

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(Manuscript received February 16, 1957)

The pleasant odors and tastes of grapes and wines, which together with the color and clarity make wines attractive beverages, are of great interest to the flavor chemist. The flavor chemist and the enologist distinguish in wines between those pleasant odors which derive primarily from the grape and those which derive from the vinification operations. The former are usually designated aroma while the latter are known as bouquet. The bouquet of wine may be considered to consist of several components among which some of the most interesting are those which are produced from non-odorous precursor compounds in the grapes. The conversion of substances of little or no odor through the action of fermentation enzymes, the slow oxidation of barrel aging, and the reducing conditions of bottle aging into bouquet substances in the mature wine is at present only very imperfectly understood. Similarly the steps in the biosynthetic pathways by which aroma materials are elaborated in maturing grapes are not fully known. In fact, our knowledge of the identities of grape aroma constituents is quite limited. It seems logical, however, that any study of bouquet composition be preceded by determination of the aroma constituents of the particular grape used for production of the wine investigated.

Three species of the genus *Vitis* have been investigated in attempts to determine the composition of the aroma. These are *Vitis labrusca, Vitis rotundifolia,* and two varieties of *Vitis vinifera,* Zinfandel and Muscat of Alexandria. In general the volatile aroma substances identified may be divided into two groups: those lower boiling, simpler, organic compounds which seem to be common to all fruit aroma distillates, and higher boiling compounds which may be characteristic of the particular species or variety. Thus, the higher boiling ester, methyl anthranilate, seems to be a characteristic essence component of the *labrusca* species *(20)* although the complete volatile essence of the Concord variety has recently been shown to contain a number of the simpler lower boiling substances and some additional higher boiling components as well *(11)*. The volatile essence from mixed varieties of *Vitis rotundifolia* similarly has been found to contain 2-phenylethanol in addition to a number of the lower boiling organic substances *(15)*.

In contrast with the pronounced characteristic aromas of the *labrusca* and the *rotundifolia,* the *vinifera* varieties in general, have delicate, subdued aromas. Also because of the great length of time during which *vinifera* varieties have been grown and selected there are large differences in aromas among the varieties. The various muscat varieties, for instance, have aromas approaching those of the *labrusca* hybrids in intensity while varieties such as the Thompson Seedless are essentially devoid of aroma material. The aromas of the White Riesling, the Semillon, and the Sauvignon blanc are all distinctly different from one another and each is readily differentiated from the muscat.

The situation with respect to the red or black *vinifera* varieties is somewhat more complicated because the more desirable of these varieties yield mature wines in which the bouquet is the overriding palate impression. The grape aromas of these varieties, while in some cases quite pronounced, are so modified by the vinification and aging operations that there is little organoleptic resemblance between the grape and wine. The white varieties are usually fermented at lower temperatures than the reds which fact tends to preserve the identity and concentration of the grape aroma material in the whites. In addition, varietal white wines usually are aged for shorter times in larger casks at cooler temperatures—all conditions tending to minimize the changes occurring in the characteristic grape aroma during the conversion of the grapes to wine.

Analysis of the aroma materials of the *Vitis vinifera* variety Zinfandel, a black wine grape, has shown the presence of a number of lower boiling organic substances, and the high boiling ester, n-butylphthalate *(8)*. The *vinifera* variety, Muscat of Alexandria, has been investigated with respect to the presence of high boiling terpene alcohols *(5)*. The research reported in this paper has been concerned with the identification of a number of the other components of the aroma of Muscat of Alexandria.

EXPERIMENTAL

The Muscat of Alexandria grapes utilized in the following experiments were obtained in the fall of 1952 partly from the Fresno area and partly from the experimental vineyards of the University of California at Davis. The fresh grapes were washed and stemmed by hand and all raisins and spoiled grapes were discarded in the process. A total of 11,155 pounds of stemmed grapes were passed through a Langsenkamp pulper to remove the seeds and skins and give a purée of the pulp and juice at approximately the rate at which the purée could be processed further. The purée was passed through a stainless steel flash evaporator *(15)* at a flow rate of about 200 ml. per minute with the evaporator operated under a pressure of 15 mm. of mercury and temperature conditions such that about 40–45% of the total volume of the purée passed over as distillate. Organoleptic analysis indicated that essentially no typical muscat aroma or flavor could be detected in the residues from the flash evaporation. The distillates from the flash evaporation were passed through an Oldershaw type distillation column using a continuous flow of about 1000 ml. per hour and 40 ml. per hour takeoff rate. Organoleptic and wet combustion analysis of the residue from the column indicated that essentially no aroma material remained in this residue. The combined concentrates (13 gallons) were passed through the column again at a feed rate of 500 ml. per hour and a takeoff rate of 50 ml. per hour. The concentrate from this second passage through the column was saturated with USP sodium chloride and exhaustively extracted with purified diethyl ether over a 70-day period with fresh extracting solvent being used every three or four days. The combined ether extracts were thoroughly dried using anhydrous magnesium sulfate. Investigation of the aqueous residue from the extraction is described subsequently.

Ether was removed from the dried ether extracts by distillation through a micro Podbielniak column under atmospheric pressure to leave a total of 554.2 g. of muscat essence. Elemental analysis after sodium fusion showed the absence of nitrogen or sulfur containing compounds in the essence. A carefully weighed amount (87.5 g.) of absolute ethanol (>99.95%) was added to the essence at this point to remove any remaining traces of water as the ethanol-water azeotrope *(22)* in the subsequent distillation. Distillation was then continued through the micro Podbielniak column over a period of several days until the remaining material in the boiler started to darken from decomposition, during which time 480 g. of essence, in addition to the added ethanol above, had been collected as distillate. The head temperature indicated that pure ethanol was coming over at this point. The above distillate was subsequently fractionally distilled through an 8 mm. by 48 inch Podbielniak column at atmospheric pressure to give Fractions 1–25

described in Part A of Table 1. Distillation was stopped at this point and the remainder of the ethanol was not further distilled and is indicated as Fraction 26.

The discolored boiler material from the first distillation using the micro Podbielniak column was flash distilled into a series of receivers cooled in dry ice baths and under a nitrogen atmosphere while the system was evacuated at the full capacity of a Welch Duo-Seal pump. The boiler was heated to a temperature of 150° C. for a few moments at the end to drive over all possible volatile material. This left about 2 g. of dark tarry residue in the boiler. This distillate was then fractionally distilled through a vacuum model Piros-Glover spinning band column, at atmospheric pressure first to remove an additional 37.5 g. of ethanol, and then under reduced pressure. The data for the reduced pressure distillation are summarized in Part B of Table 1.

Investigation of aqueous residue from ether extraction. The aqueous residue (11,725 ml.) gave a strong positive test for aldehydes with Schiff's reagent. This aldehyde was identified as acetaldehyde by preparation of the 2,4-dinitrophenylhydrazone, m.p. 161–163° C., and the dimedone derivative, m.p. 140–142° C., mixed m.p. with the acetaldehyde dimedone derivative 141–142° C. Cochromatography of the 2,4-dinitrophenylhydrazone with acetaldehyde-2,4-dinitrophenylhydrazone on a bentonite column showed that they were identical. Chromatography of the original 2,4-dinitrophenylhydrazone derivative gave evidence that acetaldehyde was the only carbonyl compound present in the aqueous

TABLE 1

Distillation of volatile essence from 1952 Muscat of Alexandria grapes

A. Atmospheric pressure distillation

Distilln. fraction	Distilln. temp., ° C.	Pressure, mm. Hg	Fraction wt., g.	Sapon. equiv.[1]	Acetyln. equiv.[2]	Test for carbonyl[3]	Test for methanol[4]	Test for unsatn.[5]
1	36.0–64.0	762	0.78	384	71	+++	+	0
2	64.0–67.4	762	1.66	1263	54	+++	+	0
3	67.4–72.0	762	1.36	1540	61	+++	+	0
4	72.0–75.0	762	1.20	1480	53	+++	+	0
5	75.0–76.0	762	0.82	1845	56	+++	+	0
6	76.0–76.3	762	1.23	2800	47	+++	+	0
7	76.3–77.1	762	2.79	2880	51	+++	+	0
8	77.1–77.5	762	1.69	2570	50	+	+	0
9–16	77.5–77.8	761	33.63	4500–10,000	47–52	++	trace	0
17–25	77.8–78.2	760	63.45	>10,000	46–52	0	0	0
26	Residue	.....	371.30	.....	.....	.....	.....	.....

B. Reduced pressure distillation

V-1	40.4–63.7	20	0.26	1737	77	+++	.....	trace
V-2	63.7–66.8	20	0.28	358	157	++	.....	++
V-3	66.8–69.4	20	0.34	677	97	+	.....	+++
V-4	69.4–71.1	20	0.90	2960	90	+	.....	++++
V-5	71.1–72.2	20	0.56	4220	94	trace	.....	+++
V-6	72.2–72.4	20	0.48	4060	90	trace	.....	++
V-7	46.6–52.2	4	0.22	4750	87	+	.....	trace
V-8	52.2–76.2	4	0.47	763	149	+	.....	0
V-9	76.2–81.6	4	0.22	649	117	+	.....	.....
V-10	81.6–83.2	4	0.32	653	130	++	.....	.....
V-11	83.2–89.1	4	0.34	586	178	++	.....	.....
V-12	89.1–94.0	4	0.26	1465	128	+++	.....	.....
V-13	94.0–96.5	4	0.31	475	157	+++	.....	.....
V-14[6]		....	0.48	384	185	.....	.....	.....
V-15	Residue	....	ca 2.0	.....	.....	.....	.....	.....

[1] Grams of sample containing one equivalent of ester *(22)*.
[2] Grams of sample containing one equivalent of alcohol *(14)*.
[3] Test for aldehydes, ketones and acetals with 2,4-dinitrophenylhydrazine *(21)*.
[4] Chromotropic acid test *(7)*.
[5] Absorbtion of 1% bromine in CCl_4 solution.
[6] Fraction V-14 was obtained by distillation of the remaining material under a nitrogen atmosphere at full vacuum pump capacity with no fractionation.

residue. A total of 3.82 g. of acetaldehyde was found to be present in the aqueous residue by gravimetric analysis as the 2,4-dinitrophenylhydrazone using the method of Iddles and Jackson *(12)*. An additional 0.49 g. of acetaldehyde was found by a similar analysis to be present in the ether which was stripped from the essence by distillation through the micro Podbielniak column.

Acetaldehyde was removed from 100 ml. of the aqueous residue by treatment with a two-fold excess of 2,4-dinitrophenylhydrazine reagent and the precipitated derivative removed by filtration. The suction flask was vented through a dry ice trap during the filtration to prevent loss of volatile materials. Precipitate was washed thoroughly with water and the filtrate then distilled through a short fractionating column for a considerable time after the head temperature reached 100° C. This distillate gave a positive test for methanol using chromotropic acid *(7)*. The distillate was analyzed by density determinations and by preparation of the 3,5-dinitrobenzoate derivatives by the methods *(10, 18)* developed for use with dilute aqueous alcohol solutions followed by chromatographic separation of the 3,5-dinitrobenzoate derivatives. From these analyses it was calculated that the aqueous residue from the ether extractions contained a total of 49 g. of ethanol and 16 g. of methanol. These substances were identified by the melting points of the 3,5-dinitrobenzoates as follows: material from top band, m.p. 107.5–108° C., mixed m.p. with methyl 3,5-dinitrobenzoate 107–108° C.; material from bottom band, m.p. 92.5–93.0° C., mixed m.p. with ethyl-3,5-dinitrobenzoate 92.5–93.0° C.

Identification of alcohols. The alcohols present in approximately 15 mg. of each distillation fraction were converted to the 3,5-dinitrobenzoate derivatives by reaction with 3,5-dinitrobenzoyl chloride in anhydrous pyridine *(22)*. The crude derivative preparation was then chromatographed *(25)*, the bands eluted and identified by means of melting points and mixed melting points with known derivatives. In some cases conversion of the 3,5-dinitrobenzoate derivative to the α-naphthylamine addition product was also utilized. The data obtained are presented in Table 2.

Identification of esters. Saponification equivalents of the various distillation fractions were determined on 5–10 mg. samples using the diethylene glycol method *(22)*. For ester identification purposes 100–200 mg. samples of the distillation fractions were saponified in either diethylene glycol *(22)* or dioxane *(15)* solution and the alcohol and acid portions isolated as described. The alcohols, including the free alcohols originally present in the fraction, were identified as the 3,5-dinitrobenzoate derivatives. The acids were converted into either the p-phenylphenacyl *(17)* or the p-phenylazophenacyl *(13)* derivatives which were then investigated by chromatography on silicic acid. The pure derivatives were identified by melting points and mixed melting points with known derivatives. The rates of travel of the p-phenylazophenacyl derivatives relative to p-phenylazophenacyl bromide, R_f values, on silicic acid *(16)* were also utilized in identifying the acids. Data from the investigation of the esters are presented in Table 3.

Identification of carbonyl compounds. Carbonyl compounds were precipitated from small samples of the various distillation fractions as the 2,4-dinitrophenylhydrazones and the crude derivative preparation in each case was investigated by chromatography on bentonite-celite mixture *(23)* or on silicic acid *(19)*. Pure derivatives were identified by melting points and mixed melting points with known derivatives and by cochromatography with known derivatives. Data obtained in the investigation of the carbonyl compounds are summarized in Table 4.

Analysis for acids. Titration data on the muscat essence before fractional distillation indicated that a very small amount of free acid was present. The total amount of acid present, however, was too small to permit isolation and identification.

DISCUSSION

The substances identified in the volatile aroma material of Muscat of Alexandria grapes are listed in Table 5. Although the data of this table were obtained from experiments on the crop of 1952, similar though less complete results were found from work on smaller amounts of grapes, approximately 800 and 5,000 pounds respectively, in 1948 and in 1950. The essence was isolated from the 1948 crop by a reduced pressure pot distillation procedure

TABLE 2

Identification of alcohols as 3,5-dinitrobenzoate esters by chromatographic separation on silicic acid

Distiln. fraction	Position of band	Relative amount [1]	M.P. of 3,5-dinitrobenzoate	3,5-dinitrobenzoate ester of	M.P. of known deriv.[2]	Mixed M.P.
1	Upper	S	104–106	Methanol	106.5–106.8	105–106
	Lower	M	87–89	Ethanol	93.0–93.2	90–92
2 to 6[3]	Upper	S	104–106	Methanol		105–107
	Lower	L	91–92	Ethanol		92–93
7	Upper	T	96–102	Methanol		102–106
	Lower	L	91.5–92.5	Ethanol		92–93
8	Upper	T	105–106.5	Methanol		105–107
	Lower	L	90–92	Ethanol		92–93
9–16	Upper	T	102–104	Methanol		104–107
	Lower	L	92–93	Ethanol		92–93
17–25	One band		92–93	Ethanol		92–93
V-1	Upper	S	54–58[4]	n-Butanol	62.8–63.0	54–60
	Lower	M	58–60[5]	3-methyl butanol	60–61	58–60
V-2	Upper	T[6]				
	Lower	M	46–49	cis-3-Hexenol	47–49	47–49
V-3	Upper	T[6]				
	Lower	L	47.5–49.5	cis-3-Hexenol		47–49
V-4	Top	ST[7]	101–105			
	Second	T	88.5–90.5	Ethanol		88–91
	Third	L	48–49.5[8]	cis-3-Hexenol		47–49
	Fourth	S	54–56	n-Hexanol	57.9–58.7	54–56
V-5	Upper	T[6]				
	Middle	S	47.5–49.0	cis-3-Hexenol		47–49
	Lower	L	53–55[9]	n-Hexanol		54–56
V-6	Upper	T	43–47	cis-3-Hexenol		43–48
	Lower	L	55–57	n-Hexanol		54–56
V-7 to V-14[3]	One band		56–58	n-Hexanol		56–58

[1] L = large, M = medium, S = small, T = trace.

[2] The melting points of known derivatives are listed only the first time each appears in the table.

[3] The results for the fractions grouped together were quite similar and typical melting point data are listed.

[4] Anal. calcd. for $C_{11}H_{12}O_6N_2$: C, 49.25; H, 4.51. Found: C, 49.60; H, 4.76. α-Naphthylamine addition compound, m.p. 89–91° C.; mixed m.p. with n-butyl-3,5-dinitrobenzoate α-naphthylamine addition compound (lit. m.p. 93° C.) was 88–90.5° C.

[5] Anal. calcd. for $C_{12}H_{14}O_6N_2$: C, 51.05; H, 4.99. Found: C, 51.28; H, 4.96.

[6] Not sufficient material for positive identification.

[7] Cochromatography and mixed m.p. data indicate that this extremely faint band was neither the methyl nor the 2-phenylethyl derivative.

[8] Anal. calcd. for $C_{13}H_{14}O_6N_2$: C, 53.06; H, 4.80; N, 9.52. Found: C, 52.33; H, 4.98; N, 9.45.

[9] Anal. calcd. for $C_{13}H_{16}O_6N_2$: C, 52.70; H, 5.44; N, 9.46. Found: C, 52.72; H, 4.92; N, 9.31.

which could reasonably have yielded considerable quantities of ethanol as a result of enzyme activity on the sugar present. It was suspected that the relatively large amounts of ethanol found in the essence had come from this source rather than being present as the alcohol within the grape. As this question could not be resolved by the 1948 experiments, essence was isolated from the larger quantity of grapes in 1950 by means of the flash evaporation technique. This technique effectively eliminated the possibility of fermentation ethanol being present in the essence. The grapes available in 1950, however, were infected with considerable quantities of several species of molds.

TABLE 3

Analysis of esters

A. Acid part

Distiln. fraction	Position of band	Relative amount [1]	M.P. of derivative		Deriv. of	M.P. of known deriv.[2]		Mixed M.P.	
			p-phenyl-phenacyl	p-phenyl-azophenacyl		p-phenyl-phenacyl	p-phenyl-azophenacyl	p-phenyl-phenacyl	p-phenyl-azophenacyl
1	One band	L	108.5–109.5		Acetic	110.5–110.8		109–110	
2	One band	S	105–108		Acetic			109–110	
V-2	One band	L	69.5–70.3		Caproic	69.5–70.0		69.5–70.3	
V-3	One band	M	65–57		Caproic			67.5–69.0	
V-8	Upper	T[3]							
	Middle	L	68.0–69.5		Caproic			68.5–69.5	
	Lower	S							
V-9	Upper	T[3]							
	Middle	M	65–66.5		Caproic			68.5–69.5	
	Lower	S							
V-8 to	Top	S		95–97	Butyric		98–99		94–99
V-11[4]	Second	T[3]		93–94					
	Third	S		68–69	Valeric		73–74		68.5–70.0
	Fourth	L		83.0–83.5	Caproic		83–84		83–84
	Fifth	S		80.5–82.0	Caprylic		83.0–83.5		81–83
V-13 to	Upper	M		80.5–82.5	Caproic				81.5–83.0
V-14[4]	Middle	M		81.5–83.5	Capric		84.5–85.5		83–84.5
	Lower	L		86.5–87.1	Lauric		87–87.5		86–87

TABLE 3

Analysis of esters

B. Alcohol part [5]

Distilln. fraction	Position of band	Relative amount	M.P. of 3,5-Dinitrobenzoate deriv.	3,5-Dinitrobenzoate ester of	Mixed M.P.
1 and 2	Upper			Methanol [6]	
	Lower			Ethanol	
V-2	Upper	M	91.5–92.5	Ethanol	91.5–93.0
	Lower	M		cis-3-Hexenol	
V-3	Upper	S	91–92.5	Ethanol	92–92.5
	Lower	M		cis-3-Hexenol	
V-8 to	Upper	ST [3]	102–105 [7]		
V-11 [4]	Middle	S	92–93	Ethanol	92–93
	Lower	L		n-Hexanol	
V-13 to	Upper	ST [3]			
V-14 [4]	Middle	S	91–92	Ethanol	91–92
	Lower	L		n-Hexanol	

[1] L = large, M = medium, S = small, T = trace.
[2] The melting points of known derivatives are listed only the first time each appears in the table.
[3] Not sufficient material for positive identification.
[4] The small amounts remaining of those fractions were combined as indicated, and the two combined samples were saponified.
[5] Data are tabulated only for those alcohols which were not previously identified as free alcohols in the samples.
[6] The amount of methanol relative to ethanol was found to be substantially increased after saponification.
[7] Mixed melting point determinations showed that this alcohol was neither methanol nor 2-phenylethanol.

TABLE 4

Identification of carbonyl compounds by chromatography of the 2,4-dinitrophenylhydrazone derivatives

Distiln. fraction [1]	Relative amount [2]	M.P. of 2,4-Dinitrophenyl-hydrazone	2,4-Dinitrophenyl-hydrazone of	M.P. of known deriv.[3]	Mixed M.P.
1 to 16	S	166.0–167.5	Acetaldehyde	167–168	166.5–168
	T	109–112[4]	2-Butanone	115.2–115.5	112–115
	T	141.5–143.0[4]	2-Pentanone	145.5–146	141.5–145
V-1 to V-3	S	165–166	Acetaldehyde		165–167
V-4 to V-9	S	165–166	Acetaldehyde		
	M	144–146[4]	2-Hexenal	146.5–147.0	144–147
V-10 to V-14	M	144.4–146.3[4]	2-Hexenal		144–147
	M	106–107[4]	n-Hexanal	107–108	106–107

[1] Results for the fractions grouped together were similar although minor differences in relative amounts were observed. Typical melting point data are listed.
[2] M = medium, S = small, T = trace.
[3] The melting points of known derivatives are listed only the first time they appear in the table.
[4] Cochromatography with the corresponding known derivative gave only one zone.

Results of analysis of the 1950 essence, therefore, were subject to some uncertainty since mold metabolites may have been present in the essence. The presence of relatively large amounts of ethanol in the essence isolated from the 1952 crop is not subject to the uncertainties troubling the earlier work, since in this later experiment the fruit was in good condition and the essence was isolated using the flash evaporator. Ethanol, therefore, must be accepted as the most plentiful component of the volatile essence of the Muscat of Alexandria grape. This finding is in general agreement with other published research on fruit volatile essences *(3, 4, 8, 9, 11, 15, 24)*.

Identification of acetaldehyde as a major component of the volatile essence is also in agreement with other research findings on fruit essences. The question of whether these two carbon compounds, which are found in so many different fruits, are present in the reported concentrations in the fruit before harvest or whether there is an accumulation of these substances in the fruit after its severance from the plant is of considerable interest. While determination of the concentrations of ethanol, acetaldehyde, and acetic acid in fruits as functions of the time between harvest and analysis could provide the answer

TABLE 5

Volatile essence components of Vitis vinifera var. Muscat of Alexandria

Component	mg. per kg. grape berries	Component	mg. per kg. grape berries
Methanol	3.7	Methyl Acetate	0.08
Ethanol	111.0	Ethyl Caproate	0.04
n-Butanol	0.03		
3-methylbutanol	0.01	Butyrate Ester	
n-Hexanol	0.49	Valerate Ester	
cis-3-Hexenol	0.26	Caproate Ester	
Acetaldehyde	0.85	Caprylate Ester	0.16
n-Hexanal	0.03	Caprate Ester	
2-Hexenal	0.05	Laurate Ester	
2-Butanone	0.01	Ethyl Esters	
2-Pentanone	0.01	Acetals	.....

to this question, the present work on the Muscat of Alexandria permits stating only that the intact fruit when analyzed a few days after harvest contains significant amounts of ethanol and acetaldehyde.

The presence of n-butanol, n-hexanol, n-hexanal and the butyrate, caproate, caprylate, caprate, and laurate esters is of interest in that it again demonstrates the tendency in plant materials toward formation of the even number carbon atom straight chain skeleton. These compounds likely arise from head to tail acetyl-coenzyme A condensations followed by reduction, dehydration, and hydrogenation. The ketone, 2-butanone, and the unsaturated aldehyde, 2-hexenal, are reasonable intermediates in the reduction, dehydration, hydrogenation mechanism considered likely for production of saturated straight chain alcohols as postulated above. The 3-methylbutanol could be produced by a similar type of reaction with the difference that the acetyl-coenzyme A condensations would be of the tail to tail type, in which a molecule of CO_2 is lost at one step in the reactions following the condensation.

The five-carbon straight chain ketone, 2-pentanone, can not be readily derived by acetyl-coenzyme A condensations and subsequent reactions as outlined above. The carbon skeleton, however, is not uncommon among natural plant substances.

The rather high methanol content, 3.7 mg. per kg., found in the muscat grapes probably represents the amount of this alcohol which exists in the fruit in equilibrium with the poly-methyl-galacturonates and the methoxylated polyphenols. Methanol also has been found frequently as a constituent of other fruit volatile essences.

Two esters were identified with certainty and a number of the others with a high degree of probability. The difficulty in unambiguous ester characterization in this work is great because many of the high boiling fractions contained mixtures of several esters as well as free alcohols. The free alcohols present may also have been present as ester components. Examination of the saponification equivalents in Table 1 shows that esters tend to occur in 4 general groups with significantly different boiling points. The ester in distillation fraction 1 is undoubtedly mainly methyl acetate but the possibility of the presence of small amounts of ethyl acetate can not be completely eliminated. The ester in fraction V-2 is ethyl caproate. Fractions V-8 through V-11, with a boiling range of 52° to 89° C. at 4 mm. Hg pressure, contain esters in which butyric, valeric, caproic, and caprylic acids are components. The 2 alcohols present in these fractions after saponification are ethyl and n-hexyl alcohols, and n-hexyl alcohol also is known to be present as free alcohol in these same fractions. Consideration of the boiling points of all possible ester combinations of the 4 acids and 2 alcohols suggests that the esters actually present are probably n-hexyl butyrate, n-hexyl valerate, n-hexyl caproate, and ethyl caprylate. It is not known, however, whether or not azeotropes of hexyl alcohol and the possible esters of the acids identified in these fractions are formed at 4 mm. Hg distillation pressure, so the identifications must remain as tentative.

Fractions V-13 and V-14, with boiling points higher than 94° at 4 mm. Hg pressure, are found to contain caproic, capric, and lauric acids as ester components. The only free alcohol present is n-hexyl alcohol, and after saponification, ethyl and n-hexyl alcohols are found. Boiling point considerations

suggest that the esters present are n-hexyl caproate, ethyl caprate, and ethyl laurate, but again, as there no data relative to azeotropes available at this pressure the identifications are to be considered tentative.

In the chromatographic analysis of the alcohol derivatives of fraction V-4 when a very large sample of the fraction was used for preparation of the derivatives, very small amounts of the 3,5-dinitrobenzoate of an unknown alcohol, M.P. 101–105° C., were found. Mixed melting point and cochromatography data show that this alcohol is neither the methyl nor the 2-phenylethyl derivative. The position of the band on the chromatographic column also shows that it is not the benzyl alcohol derivative *(1)* but suggests that it may be the derivative of either a highly unsaturated aliphatic alcohol, an alicyclic alcohol such as a terpene *(2)* or an unsaturated aromatic alcohol such as cinnamyl. The data indicate, however, that it is not one of the terpene alcohols identified in Muscat grapes by Cordonnier *(5)*.

The presence of significant amounts of cis-3-hexenol (leaf alcohol) in fractions V-2 through V-6 accounts for the "green" or grassy aroma of the muscat essence. Leaf alcohol has been identified as a constituent of several fruit essences. The configuration and the position of the unsaturation in the muscat hexenol are confirmed by comparison of 3,5-dinitrobenzoate melting points and mixed melting points and by cochromatography with the derivative of hexenol from Japanese peppermint oil, which has been shown conclusively to be cis-3-hexenol *(6)*.

The compound, 2-hexenal, also has a "green" or grassy smell. The geometrical configuration of the particular hexenal in the muscat essence was not established, but mixed melting point data and cochromatography with the 2,4-dinitrophenylhydrazone of an authentic 2-hexenal indicated that the unsaturation was in the two position.

The finding of acetaldehyde in fractions V-1 through V-9, where the boiling points are significantly higher than that of free acetaldehyde, indicates that the aldehyde was not present as free acetaldehyde when fractionally distilled. It is significant also that in fractions V-4 through V-7 the acetylation equivalent weights range from 87 to 94 although the equivalent weights of the only two alcohols present in appreciable amounts are 100 for the leaf alcohol and 102 for the n-hexyl alcohol. The two foregoing observations support the belief that fractions V-4 through V-7 (and possibly V-1, V-2, V-8, and V-9 as well) contain acetals resulting from reaction of acetaldehyde with 3, 4, or 5 carbon alcohols. It is known that acetals are hydrolyzed when treated with the highly acidic carbonyl derivatizing reagent, and it seems likely also that they would be hydrolyzed under the conditions of the quantitative acetylation equivalent determination. It is unfortunate that the residues from the acetylation equivalent determinations were not checked for the presence of alcohols of 3, 4, and 5 carbon atoms.

In an investigation of the essences of Muscat of Alexandria and of Muscat de Frontignan by chromatographic methods, Cordonnier *(5)* obtained evidence for the presence of geraniol, terpineol, limonene, and linaloöl, in a total concentration of approximately 2 mg. per liter of juice or wine. These compounds were not detected during the course of the work being reported, possibly because they were decomposed during the long time of heating in the still pots during the early fractional distillations. It is anticipated that

dehydration and polymerization reaction products of these 4 substances may be found in fraction V-15 which is still under investigation.

SUMMARY

The composition of the volatile essence from *Vitis vinifera* var. Muscat of Alexandria has been determined. A total of 11,155 pounds of grape berries were processed and the volatile essence components isolated.

The volatile essence was found to consist of methanol, ethanol, n-butanol, 3-methylbutanol, n-hexanol, cis-3-hexenol, acetaldehyde, n-hexanal, 2-butanone, 2-pentanone, 2-hexenal, methyl acetate, ethyl caproate, a butyrate ester, a valerate ester, another caproate ester, a caprylate ester, a caprate ester, a laurate ester, ethyl esters, and acetals. The concentrations of these components in the grape berries were estimated. Possible modes of formation of a number of these components were discussed.

Acknowledgment

The authors are pleased to record their gratitude to J. W. Gates, Jr. of the Eastman Kodak Company for a sample of 2-hexenal, and to S. H. Harper of King's College for the sample of cis-3-hexenol-3,5-dinitrobenzoate. Financial support by the Wine Advisory Board is also gratefully acknowledged.

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FURTHER STUDIES ON BACTERIAL ENDOSPORE GERMINATION RELATIVE TO AUTO-INHIBITION [a]

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(Manuscript received February 20, 1957)

The induction of bacterial endospore germination in simple aqueous solution by a variety of metabolic substances, alone and in various combinations, is well known. Systems of this type have been used extensively to investigate biochemical changes during the transition from spore to vegetative cell; details of current knowledge of this subject have been recently reviewed by Stedman *(9)*. Since the publication of this review, a previously undescribed phenomenon termed "auto-inhibition" has been shown in this laboratory to occur during germination of the spores of *Bacillus globigii (10)*. In brief, it was reported that spores initially germinating in L-alanine and glucose solutions secrete an inhibitory substance which interferes with the germination of the remaining spores in the population. The present communication describes further studies on the induced germination of *B. globigii* and the influence of auto-inhibition on the germination pattern.

EXPERIMENTAL

Bacillus globigii, Fort Detrick strain, was used throughout the work. The organism was grown on "G" agar *(1)* fortified with 1% casein hydrolysate (dry weight basis) for 4–5 days, at which time microscopic observation showed that sporulation was complete. The spores were harvested, washed using the procedure of Stewart and Halvorson *(11)* and stored as concentrates in the freezer (−25° C.). Spore suspensions stored for more than 2 weeks were discarded to avoid "aging" effects *(2)*.

In the germination induction experiments, concentrates were thawed, diluted in 0.067 M phosphate buffer (pH 7.0) to a concentration of 1 mg. dry weight/ml., and heat-shocked at 65° C. for 15 minutes. Each tube contained glucose and L-alanine as inducers, 1 mg. spores, phosphate buffer and other substances (see Results) in a total volume of 5.0 ml., as described in a previous report *(10)*. In a few instances, 0.05 M acetate buffer was substituted for the phosphate buffer; both buffers give essentially the same patterns of germination with alanine and glucose, although acetate is slightly slower than phosphate. Percentage germination was determined by the turbidimetric method of Hachisuka *et al. (3)* and occasionally confirmed by standard procedures involving uptake of methylene blue and loss of heat resistance *(9)*.

In some instances kinetic analyses were performed by measuring the percentage germination in the presence and absence of specific exogenous inhibitors using the above germination induction method and by evaluating the data using the double reciprocal plot [e] of Lineweaver and Burk *(5)*. In all cases, the maximum rate of germination occurred in the 10–20 minute interval after addition of inducers and inhibitors. Arbitrarily, the

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[a] The views expressed herein are those of the authors and are not necessarily similar to the views of the U. S. Department of the Navy.

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[e] In this technique, a plot of the reciprocal of the substrate concentration vs. the reciprocal of the reaction velocity is made. The specificity of the inhibition, i.e. whether competitive or non-competitive, can be readily determined from the resulting figure.

percentage of spores germinated in this interval was considered the velocity constant, V, and reciprocals calculated accordingly.

RESULTS

As noted previously *(10)*, the presence of the auto-inhibitory substance secreted by germinating spores can be detected in two ways: after the rate of germination has leveled off, replacement of the liquid medium by fresh buffer and inducers causes a resumption in the germinating rate; and, addition of the medium in which spores have previously germinated to a fresh spore suspension containing inducers retards markedly the rate of germination observed therein. The manner in which this retardation occurred was suggestive of a competitive type inhibition. Accordingly, an analysis of the kinetics of the inhibition was initially attempted in the present study.

All tubes contained spores, phosphate buffer, 10 μM glucose and L-alanine with or without added auto-inhibitory substance. Three levels of L-alanine were employed; 10, 50 and 250 μM. Auto-inhibitory substance was prepared by pooling the supernatants from a number of tubes in which germination of spores had leveled off after induction by L-alanine and glucose. This pooled supernatant, containing the auto-inhibitory substance, was used undiluted, diluted 1:2 and diluted 1:3 with buffer in the trials. Nine possible combinations of L-alanine and auto-inhibitory substance were thus obtained. The patterns of germination resulting from these combinations were then analyzed as indicated above. The results are shown in Figure 1. A typical competitive inhibition is evident.

Since a number of substances have been shown to be excreted during endospore germination, including amino acids, a number of the latter compounds were tested for inhibition of L-alanine induced germination. Consistent

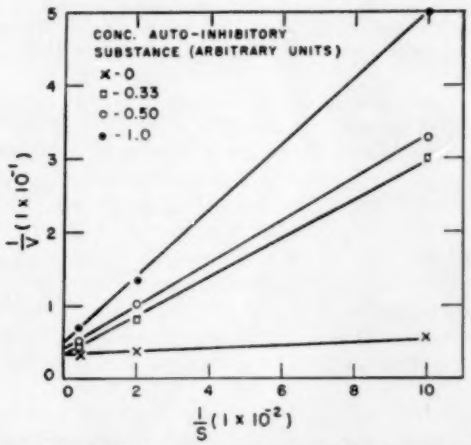

Figure 1. Competitive inhibition of L-alanine induced endospore (B. globigii) germination by the auto-inhibitory substance. Each tube contained 1 mg. dry weight spores, 5 ml. phosphate buffer, L-alamine (10, 50 or 250 μM) and "auto-inhibitory substance." Abcissa refers to L-alanine concentrations. "S" refers to the concentration of L-alanine; "V" is the velocity of the germination (see text).

inhibition of germination was found only with DL-serine, although L-tyrosine also gave inhibition in most cases. Varying results were obtained with the following amino acids used at a 250 μM level in a succession of daily experiments: L-aspartic acid, DL-citrulline, L-cysteine, L-glutamic acid, glycine, L-phenylalanine, L-lysine, DL-methionine, DL-norleucine, DL-ornithine, L-threonine, L-valine, L-proline, DL-histidine, L-arginine, L-leucine and L-isoleucine. In no instance was a "reversal" of the auto-inhibitory effect evident, i.e. 100% germination was never obtained.

Next, it was desired to determine whether DL-serine interfered with L-alanine induced germination in a competitive manner as the auto-inhibitory substance does. An inhibition analysis was performed under the same conditions as above except that 10, 20 and 30 μM, DL-serine were substituted for the auto-inhibitory supernatants. The results are shown in Figure 2.

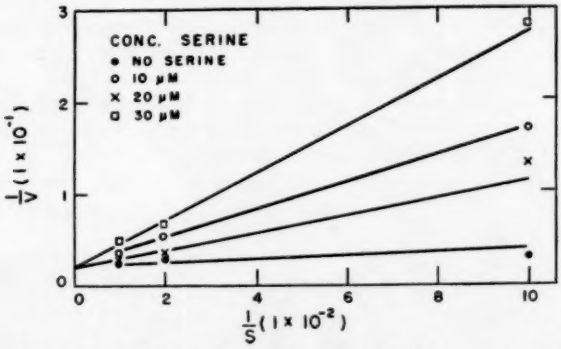

Figure 2. Competitive inhibition of L-alanine induced endospore (B. globigii) germination by DL-serine. Conditions were similar to Figure 1 except DL-serine was substituted for "auto-inhibitory substance." See Figure 1 for explanation of coordinate scales.

Again, a typical competitive inhibition is evident. Thus, DL-serine competes directly with L-alanine at the site of the "triggering" reaction which initiates germination, in a manner similar to the auto-inhibitory substance.

Since the competitive inhibition of L-alanine action by DL-serine suggested a structural antagonism, effort was devoted to determining the effect of two other C_3-carboxylic acids of known importance in oxidative metabolism: lactate and pyruvate. Both of these compounds showed an antagonism of L-alanine inductive capacity similar to DL-serine on initial trials. Accordingly, inhibition analyses of lactate and pyruvate were also performed. Pyruvate exhibited competitive inhibition in the same fashion as DL-serine. On the other hand, lactate demonstrated a non-competitive type of inhibition, as shown in Figure 3.

These findings show that DL-serine, pyruvate, and possibly other amino acids and metabolic compounds are capable of playing a specific role in interfering with the inductive effect of L-alanine on bacterial endospore germination.

Since dipicolinic acid (pyridine-2,6-dicarboxylic acid) is present in significant amounts in bacterial endospores *(4, 9)* and is secreted during germination

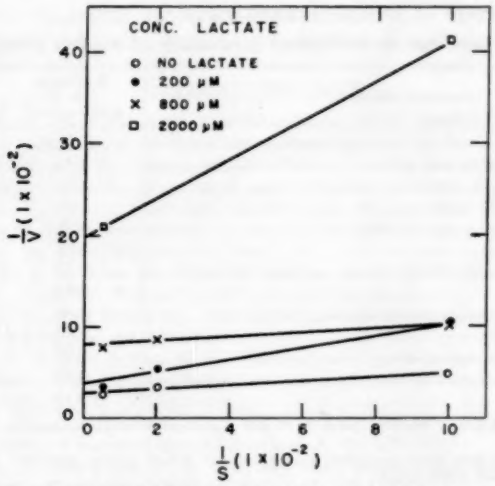

Figure 3. Non-competitive inhibition of L-alanine induced endospore (B. globigii) germination by lactate. Conditions were similar to Figure 1 except lactate was substituted for "auto-inhibitory substance."

(4, 7) the effect of this compound on the inductive capacity of L-alanine was next examined. In the presence of 50 μM L-alanine and 10 μM glucose, 83% of the spore population (1 mg./tube) were germinated in 60 minutes, at which time the rate of germination had leveled off. In the presence of 5 μM dipicolinic acid, no measurable germination occurred even after 75 minutes. On the addition of 5 μM calcium ion to tubes containing 5 μM dipicolinic acid plus inducers, 69% germination was obtained in 60 minutes, showing that Ca^{++} antagonizes to a large extent the inhibition by dipicolinic acid. Calcium itself has only a slight inhibitory effect on L-alanine induced germination (see below) and does not eliminate auto-inhibition, showing that the latter effect is not due to dipicolinic acid. This relationship between Ca^{++} and dipicolinic acid is especially interesting since Ca^{++} is also secreted by endospores during germination and is believed to be combined with DPA intracellularly in the spore *(9)*.

Inorganic ions, alone and in combinations have been shown to affect significantly the germination patterns obtained with various inducers *(6)*; however, no data are available on systems in which auto-inhibition was known to exist, mainly because the latter effect has been only recently demonstrated. Accordingly, the course of germination was followed with *B. globigii* in the presence of 50 μM L-alanine, 10 μM glucose and 3 levels (50, 100 and 250 μM) each of $CaCl_2$ $MnCl_2$, $CoCl_2$ and $ZnSO_4$. Acetate buffer was substituted for the usual phosphate buffer to eliminate precipitation of the metallic ions. Table 1 gives the maximum rate of germination, expressed as percentage of the total spore population germinating per minute based on the fastest 10-minute interval, and the total percentage obtained at the end of the experiment. All ions give varying degrees of inhibition of germination and show no reversal of the auto-inhibitory substance. Ca^{++} is the least inhibitory to

TABLE 1

Effects of inorganic ions on the induced germination of Bacillus globigii endospores

Inorganic additions [1]	Maximum rate of germination [2]	Final percentage germination [3]
0	2.0	48
50 μM $CaCl_2$	1.4	45
100 μM $CaCl_2$	1.3	45
250 μM $CaCl_2$	1.3	46
50 μM $CoCl_2$	0.91	28
100 μM $CoCl_2$	0.27	17
250 μM $CoCl_2$	0.32	5.3
50 μM $MnCl_2$	0.96	33
100 μM $MnCl_2$	0.48	23
250 μM $MnCl_2$	0.62	19
50 μM $ZnSO_4$	0.70	23
100 μM $ZnSO_4$	0.42	17
250 μM $ZnSO_4$	0.16	5.5

[1] All tubes had 1 mg. dry wt. spores + 50 μM L-alanine + 10 μM glucose + other additions as indicated.

[2] Percentage of total spore population germinating per minute during maximum rate of germination (usually 10–20 minute after start).

[3] Percentage germination after 120 minutes.

germination, and no difference is evident between the 3 concentrations tested. Zn^{++} is the most inhibitory.

DISCUSSION

The foregoing studies provide some background data for an experimental approach to isolating the auto-inhibitory substance. Although none of the findings provides conclusive evidence of a relationship to auto-inhibition, the role of serine, pyruvate or some structurally related compound seems a strong probability. Since the auto-inhibitory substance is partially heat-labile *(10)*, identity of this substance with free serine is not probable. However, the possibility exists that a terminal serine residue combined with other specific functional group or groups in a high molecular weight, heat-labile substance may be acting as the auto-inhibitory agent. Along this line, glucosamine-peptide combinations have been recently isolated from spores *(8)*. Perhaps a number of components of excreted spore substances each having different degrees of heat-lability contributes to the overall auto-inhibitory effect. In any event, isolation and identification studies are required to establish conclusively the bio-chemical nature of auto-inhibition.

SUMMARY

An extension of a previous investigation in which auto-inhibition of endospore germination was initially demonstrated has been presented. Kinetic studies have shown that the auto-inhibitory substance(s) act competitively against the germinating effects of L-alanine and glucose. DL-serine and pyruvic acid also can be demonstrated to inhibit competitively in the same way as the auto-inhibitory substance. Lactic acid inhibits non-competitively. Dipicolinic acid is strongly inhibitory to L-alanine induced germination, but the auto-inhibitory substance is not identical with this compound. The actions of certain inorganic ions known to have effect on spore germination were also

studied under conditions in which auto-inhibition is operative. Calcium, manganese, cobalt and zinc cannot reverse the action of the auto-inhibitory substance.

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MICROWAVE ENERGY IN FREEZE-DRYING PROCEDURES

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(Manuscript received February 25, 1957)

Many materials are not amenable to freeze-drying because of their dimensions or structure. For example, as beefsteak or bone tissue is freeze-dried, the ice surface at which sublimation occurs steadily recedes behind insulating dried substance. As a consequence, it becomes increasingly difficult to apply the energy for sublimation at the vaporizing surface without heat damage to the dried substance. The process accordingly is characterized by long drying cycles and small capacities when compared with the usual demand for the product. Bone, e.g., even in sections 2 cm. by 4 cm. by 8 to 10 cm., requires 8 days in a typical process *(1)*. Beefsteak has been freeze-dried in a 24-hour process *(4)*. These results were obtained with circulating water or infrared irradiation as the source of heat, and still other existing processes depend on the withdrawal of heat from the equipment ambient. The present paper describes the use of microwave energy to supply the heat of sublimation in the receding ice volume. The penetration of this type of electromagnetic energy into food has been described by Copson *(2)*. Thermal effects ten centimeters inside homogeneous materials were demonstrated.

EXPERIMENTAL

A microwave cavity using magnetron generated power at 2450 mc. was fitted with a 6-station manifold constructed of copper tubing. The manifold was connected by a short conductor to a refrigerated condenser outside of the high frequency field. A 2-stage rotary oil vacuum pump was used to exhaust the system. The condenser was maintained at −70° C. Pressure was measured with a McLeod gauge and maintained below 100 μ. The metallic manifold served to shield the sublimed vapor molecules from the electromagnetic field and to transport them from the attached vacuum desiccators to the condenser.

Operation of the device involved evacuating the system to 100 μ or less, then applying microwave energy at a fixed power level. Initially, this was 0.21 kw. seconds per gram of moisture in the raw material. At the end of the drying period the rate had increased to 1.39 kw. seconds per gram of moisture. The energy was applied at a rate sufficiently slow to allow the sublimed moisture to diffuse out of the sample, since it appeared that the application of microwave energy was limited by the maximum rate of diffusion of the vapor. The progress of freeze-drying was followed by observing the change in weight of the samples during the process.

Previous studies at lower frequencies resulted in the occurrence of glow discharge at unspecified low pressures *(3)*. This was also demonstrated in the present study at 2450 mc., but was avoided by eliminating corona discharge points, use of large moisture vapor conductors, and an efficient condensing system.

RESULTS

Results of microwave freeze-drying are shown in Figure 1. A two-thirds reduction in drying time for samples of beefsteak 2 x 0.75 x 0.75 in. (2 ounces) with the application of microwaves is indicated. This is not to be construed as a comparison with conventional freeze-drying methods where heat conduction is employed, but to demonstrate the acceptance of microwave energy by the product and its conversion to heat of sublimation. The curve showing

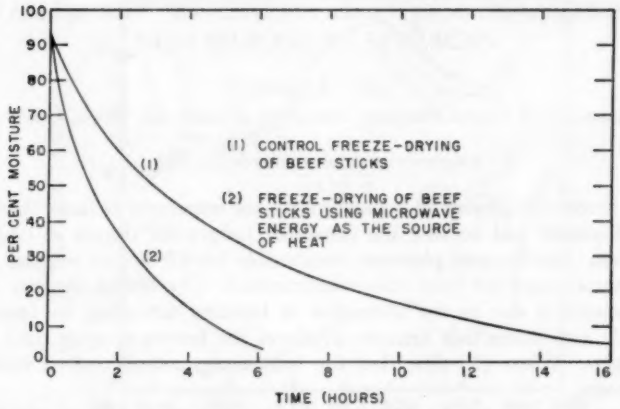

Figure 1. Comparison of rate of freeze-drying of beef sticks with and without microwave energy at 2450 mc. as a source of energy to provide the heat of sublimation.

the rate of freeze-drying without microwave energy was obtained in the same equipment, except that the magnetron was not energized. The finished product compared favorably in color, taste, and texture with the conventionally freeze-dried meat and with the fresh material, but was somewhat drier in taste than the fresh product. The utilization of available microwave power tube output was considerably sub-maximum, and efficiency determinations at normal power levels will be the subject of a later report.

DISCUSSION AND CONCLUSIONS

A uniform radio frequency heating is obtained in freeze-drying beefsteak by the application of microwave energy at 2450 mc.

The use of microwave energy permits the rapid dehydration of certain products not easily handled by conventional processes. Heat is supplied directly to the inner ice volume because of the penetrating nature of energy at this frequency. Thus products such as cuts of meat, can be dried in much less time than formerly possible and without loss in quality. It is anticipated that favorable results with bone, and other tissues, and similar heat sensitive materials will be obtained.

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REFLECTANCE SPECTRAL STUDIES OF THE HEMATIN PIGMENTS OF COOKED BEEF

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(Manuscript received March 21, 1957)

The hematin pigments of cooked meats are important because they are an index of quality and because the consumer judges the degree of cooking by their color. The hematin pigments responsible for the brown or gray color of cooked meats have not been fully characterized. The brown or gray color of cooked meats is due to the formation of hematin according to Jensen *(2)*. Lowe *(4)* also states that hematin produces the brown or gray color of well done meats. Watts *(6)* describes the brown pigment of cooked meats as a hemichrome.

Recent research *(1)* on the pink pigment of canned tuna shows that this pigment consists of a hemochrome derived from the reaction of the heme of myoglobin and residual hemoglobin with denatured globin and nicotinamide to form a mixed denatured globin nicotinamide hemochrome. Since the composition of tuna is similar to the other meats, including beef, pork and chicken, it was interesting to determine if these results apply generally to all cooked meats.

Although the use of reflectance spectrophotometry is potentially very useful for measurement, identification and differentiation of hematin pigments, this technique has been applied but little in the study of meat pigments. For the non-extractable pigments, such as those of cooked meats, reflectance spectrophotometry is probably the most useful tool for characterization without the danger of inducing artifacts.

It is the purpose of this paper to illustrate the usefulness of reflectance spectrophotometry in studying hematin pigment changes in the cooking of beef and also to characterize the hematin pigments of cooked beef.

EXPERIMENTAL

Choice beef tenderloin steaks were broiled in an oven preheated to 350° F. to final interior temperature of 150° F. and 180° F. to give steaks of degrees of doneness of medium rare and well done. Slices of the raw steak controls and these cooked steaks were used for reflectance spectrophotometry. The pigments of cooked beef steaks were reduced by brushing on solutions of sodium hydrosulfite. Some of the reduced cooked beef was exposed to gaseous carbon monoxide and some was treated with aqueous sodium cyanide. The entire visible spectra from 400 mμ to 600 mμ was recorded, using a Beckman DR recording spectrophotometer with a reflectance attachment.

RESULTS AND DISCUSSION

Typical reflectance spectra are given in Figure 1 which is a composite figure in which the spectral curves have been plotted on three ordinate scales so that the principal characteristics can be shown on the same abscissa scale. In Table 1 the main spectral characteristics of the beef pigments are compared with the main characteristics of absorbance spectra of the pure pigments.

In rare cooked beef the pink pigment lying adjacent to the brown pigment of beef appears by eye to be either oxymyoglobin or a hemochrome. This

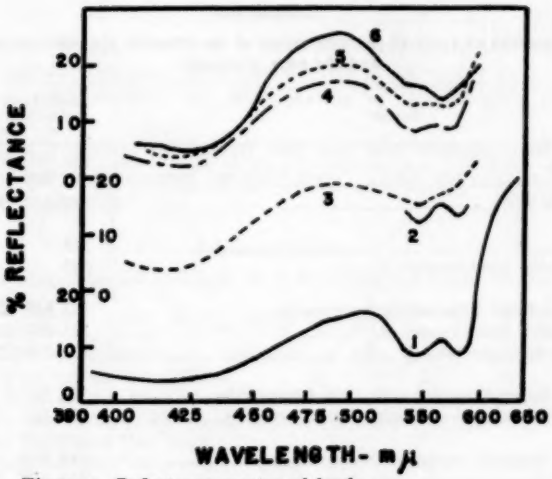

Figure 1. Reflectance spectra of beef:

1. **Fresh uncooked beef**
2. **Rare cooked beef**
3. **Well done cooked beef**
4. **Reduced cooked beef treated with carbon monoxide**
5. **Reduced cooked beef treated with cyanide**
6. **Reduced cooked beef**

pigment in the partially cooked beef could theoretically be a denatured globin hemochrome formed under reducing conditions by the heat denaturation of globin and its subsequent reaction with the heme from oxymyoglobin. However the experimental evidence (curve 2 in Figure 1 and Table 1) shows that the spectral characteristics are those of oxymyoglobin and that this pigment consists of undenatured oxymyoglobin.

The spectra of the normal brown pigment of cooked beef shows that this hematin pigment is a hemichrome having the same spectral characteristics as denatured globin hemichrome and other hemichromes. Based on the analogous studies of cooked tuna and the knowledge that denatured globin and nicotinamide are the main hemochrome forming nitrogenous compounds in meats which are available to react with the heme nucleus of myoglobin, it is proposed that the brown pigments of cooked beef are mixed denatured globin nicotinamide hemichromes. The preparation of derivatives of these hematin pigments of cooked beef and a comparison of spectral characteristics with known hemichrome derivatives was used to test this hypothesis.

When the surface of cooked beef is reduced by brushing on sodium hydrosulfite the pink hemochromes are formed. Their spectral characteristics are very similar to those of denatured globin hemochrome and nicotinamide hemochrome as shown in Table 1. These hemochromes readilly oxidize in air back to the brown hemichromes as do those of tuna. When cooked beef is reduced and subsequently treated with carbon monoxide the main spectral characteristics of the hematin pigments are identical with those of denatured globin carbon monoxide hemochrome. Likewise, when cooked beef is reduced

TABLE 1

Comparison of spectral characteristics of the hematin pigments of beef and the pure pigments

Source	Reflectance minima or absorbance maxima, mμ
Raw beef	582, 545, 418
Rare beef	580, 545
Oxymyoglobin *(3)*	582, 544, 418
Cooked beef	547
Denatured globin hemichrome *(3)*	545
Cooked beef reduced with sodium hydrosulfite	563, 535, 422
Denatured globin hemochrome *(3)*	555–560, 528–530, 424
Nicotinamide hemochrome *(1)*	555–558, 523–525, 423
Cooked beef, reduced, treated with carbon monoxide	570, 542
Denatured globin carbon monoxide hemochrome *(3)*	571, 542
Cooked beef, reduced, treated with cyanide	568, 543
Denatured globin cyanide ferroporphyrin *(3)*	568, 540

and treated with cyanide the main spectral characteristics are essentially identical with those of denatured globin cyanide ferroporphyrin.

Since all of these derivatives of the hematin pigments of cooked beef are essentially identical with the corresponding derivatives of the pure pigments and because denatured globin and nicotinamide are the main hemochrome formers present in beef, it must be concluded that the hematin pigments of cooked beef are best characterized as mixed denatured globin nicotinamide hemichromes.

The hematin pigments of cooked pork and chicken were likewise studied by reflectance spectra. The brown hematin pigments had a typical hemichrome spectra and reduction with sodium hydrosulfite gave a red pigment having a typical hemochrome spectra and the same general properties as the hemochromes of cooked beef. Therefore, the hematin pigments of cooked pork and chicken can likewise be chraacterized as denatured globin nicotinamide hemichromes. Because the results of these studies are so similar they will not be given in further detail here. Considering the similarity of hematin pigments of cooked tuna, beef, pork and chicken it is probable that the brown pigment of all cooked meat has similar composition.

Besides the undesirable reddening of cooked meats caused by the presence of nitrite or the presence of carbon monoxide there are cases of reddening of cooked meats unexplainable in terms of present knowledge *(2)*. This research shows that a simple reduction of the brown hemichromes will give the red hemochromes and it is probable that this is another cause of reddening of cooked meats.

The exact chemical nature of the red pigment formed by roasting meats *(2)* and poultry *(5)* in the presence of carbon monoxide has not been determined. This research shows that a similar red pigment best characterized as denatured globin carbon monoxide hemochrome (Table 1) results when cooked meat is exposed to carbon monoxide under reducing conditions.

SUMMARY

The hematin pigments of meats have relatively sharp and characteristic reflectance spectral minima. Reflectance spectrophotometry is a very useful technique for measurement and identification of the hematin pigments in meats.

The brown pigments of cooked beef are best characterized as mixed denatured globin nicotinamide hemichromes. Their conversion to various hemochromes is discussed.

Acknowledgments

The author appreciates the assistance of Ruth Martin and Duane Brown.

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THE RED PIGMENT OF PRECOOKED IRRADIATED MEATS [a]

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(Manuscript received March 21, 1957)

When precooked meats are irradiated, the normal brown or gray hematin pigments are converted to uncharacteristic red pigments. This color change has been noted by previous investigators *(3)*. This color change is undesirable because the consumer judges the degree of doneness of cooked meat by its color.

Recent studies in this laboratory have characterized the pigments of cooked meats using reflectance spectra as one of the primary methods *(2, 7)*.

It is the purpose ofthis paper to characterize and identify the red pigments of precooked irradiated meats.

EXPERIMENTAL

Beef, pork, chicken and tuna were cooked at 10 p.s.i. for 10 to 15 min. to develop a characteristic brown or gray color of the cooked meat. These meats were irradiated in No. 2 cans and nitrogen atmospheres at the Materials Testing Reactor, Arco, Idaho with a dose of 2×10^6 r.e.p.

The complete visible reflectance spectra of the samples was recorded using a Beckman DR recording spectrophotometer with reflectance attachment. The unirradiated controls were reduced by painting on a solution of sodium hydrosulfite.

RESULTS AND DISCUSSION

Typical spectra for beef, pork, chicken and tuna are shown in Figures 1, 2, 3, and 4, respectively. All of the spectral curves for the cooked meats are typical of denatured globin hemichrome. Upon reduction with sodium hydrosulfite each of these meats develops a double banded spectra which is characteristic of denatured globin hemochrome. In denatured globin hemochrome and the reduced pigments of cooked meats in general, the spectra show a strong α peak at about 560 mμ and a secondary β peak at 530–535 mμ *(2, 7)*. This α peak is less pronounced in beef than in pork, chicken and tuna. The precooked irradiated meats all have a double banded spectra with α and β peak at approximately the same wave length as denatured globin hemochrome except that the α and β peaks are of the same magnitude. Actually these reflectance spectra of precooked irradiated meats show greater correspondence with derivatives of denatured globin hemochrome such as denatured globin carbon monoxide hemochrome than with denatured globin hemochrome itself. There is no reason to postulate, however, that the irradiation of precooked meats forms a mixed hemochrome more complicated than that of reduced cooked meats because none of the compounds characteristically forming mixed hemochromes having these spectral characteristics, have been found in irradiated meats *(6, 8)*. These red pigments of irradiated precooked beef, pork,

[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been approved for publication. The views and conclusions contained in this report are those of the author. These are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

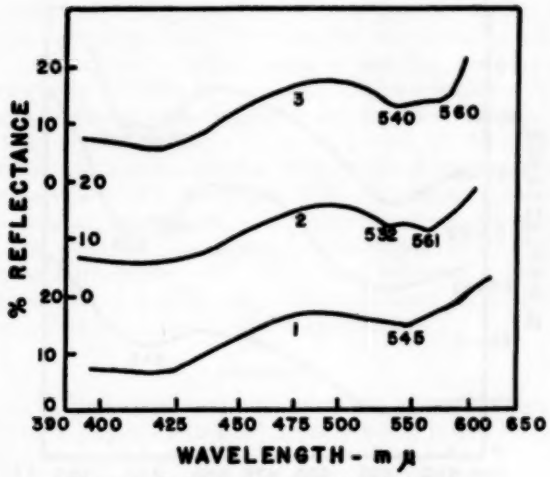

Figure 1. Reflectance spectra of cooked beef:
1. Untreated
2. Reduced with sodium hydrosulfite
3. Irradiated at 2×10^6 r.e.p.

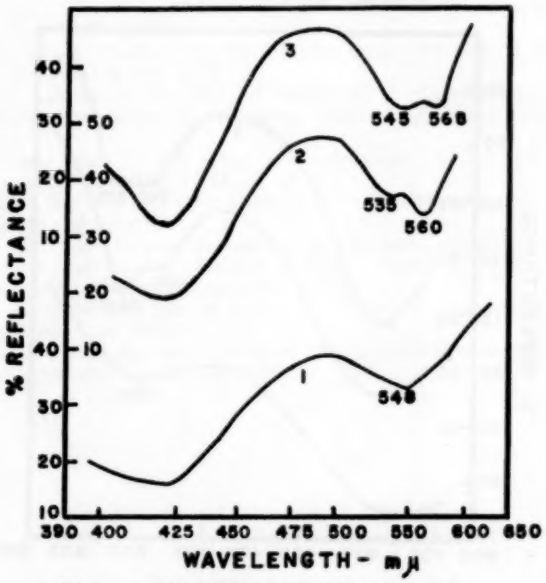

Figure 2: Reflectance spectra of cooked pork:
1. Untreated
2. Reduced with sodium hydrosulfite
3. Irradiated at 2×10^6 r.e.p.

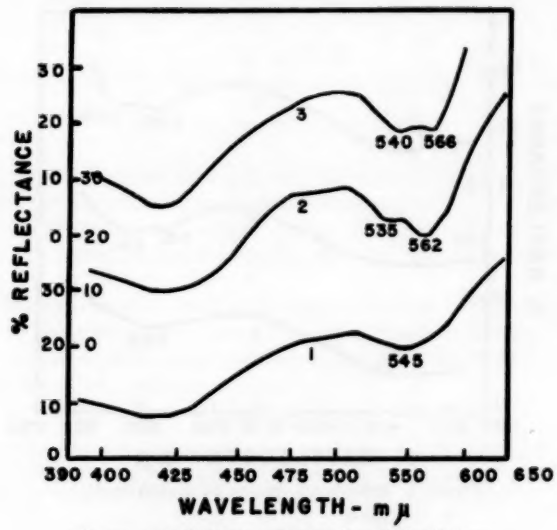

Figure 3. Reflectance spectra of cooked chicken:
1. Untreated
2. Reduced with sodium hydrosulfite
3. Irradiated at 2×10^6 r.e.p.

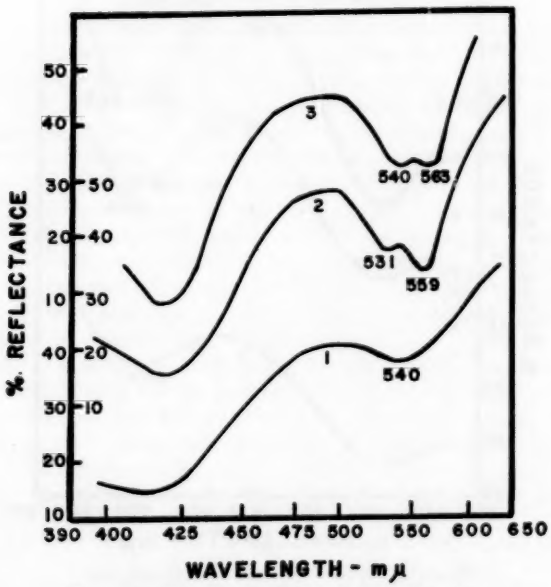

Figure 4. Reflectance spectra of cooked tuna:
1. Untreated
2. Reduced with sodium hydrosulfite
3. Irradiated at 2×10^6 r.e.p.

chicken, and tuna showed typical hemochrome oxygen lability and readily oxidized to brown hemichromes. Therefore, the best characterization of these red pigments of precooked irradiated meats is that they are denatured globin hemochromes slightly modified by irradiation to give a reduction of the α peak.

It has been previously established that irradiation of hematin compounds in oxygen free solution produces reduction as one of the main reactions. Ferricytochrome c in aqueous solution in the absence of oxygen is efficiently reduced by ultra-violet and x-irradiation *(1, 4, 5)*. This hemichrome is readily reduced both in pure solution and in the presence of organic molecules such as benzoate and succinate *(4)*. Cyanide hemichrome is also reduced to a hemochrome by x-irradiation *(1)*. The gamma radiation induced reduction of the denatured globin hemichromes of cooked meats to the corresponding hemochromes is a further extension of this generality.

SUMMARY

Irradiation of precooked meats converts the normal brown denatured globin hemichrome pigment into a red pigment which is best characterized as denatured globin hemochrome.

Acknowledgment

The author appreciates the assistance of Herman Groninger.

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A RAPID METHOD FOR THE ESTIMATION OF THE BACTERIAL CONTENT OF PRECOOKED FROZEN FOODS

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(Manuscript received March 25, 1957).

It is generally recognized that the production of wholesome precooked frozen foods requires strict control of microbial contamination and growth. For most effective control, prompt detection of sources and degree of contamination is essential. Yet 2 to 3 days are normally required to obtain bacterial counts by the usual plating methods. There is a considerable need, therefore, for a rapid method for estimating bacterial populations in the large and expanding field of precooked foods .

Rapid methods which require only a few hours for completion and which involve the reduction of methylene blue and resazurin have been developed and are standard procedures for the assay of bacteria in milk *(1)*. Attempts to apply these reductase tests to other foods have met, usually, with only partial success. Proctor and Greenlie *(6)* obtained only a very rough correlation between numbers of bacteria and resazurin reduction times in the case of fresh and frozen meats, eggs and vegetables. Also, the best results were obtained when the bacterial contamination was abnormally large. Johns *(4)* reported difficulty in determining end points and also excessively long reduction times when resazurin and methylene blue tests were applied to egg powder. Likewise, Hirschmann and Lightbody *(3)* found that the relationship between the plate counts of liquid egg material inoculated with *Pseudomonas fluorescens* and resazurin reduction time is disrupted by lyophilization and storage. Scott and Gillespie *(8, 9)*, however, obtained good correlation between plate counts and rates of resazurin reduction with egg pulp; this method is now widely used in Australia for the grading of egg mélange. Recently, Novak, Fieger, and Bailey *(5)* published a short report in which they indicated that acid formation or methylene blue reduction could be used to detect gross bacterial contamination of shrimp and oysters.

In the present paper, a reliable resazurin reduction method is described which permits the estimation of low, intermediate, and high levels of bacterial contamination in precooked frozen foods (poultry and meat pies). An important feature of the method is the addition of a culture medium to the food sample which stimulates bacterial growth and thus shortens the time necessary for the reduction of the resazurin. Results are available within a maximum of five hours after incubation of the test mixture.

METHODS

On the basis of a detailed investigation of many factors such as type and amount of indicator, composition and pH of the culture medium, temperature of incubation, preparation and size of food sample, etc., which will be discussed later, the following standard procedure was adopted and applied to 77 poultry and meat pies.

Preparation of sample. The top layer of dough is removed aseptically from the frozen pie and discarded. The entire internal contents of the pie are cut into pieces with a sterile knife and transferred with sterile forceps to a weighed sterile aluminum blender

jar equipped with a screw type cap. The blender is then reweighed and the increase is the weight of the pie material. Sterile 0.1% peptone water is added to the blender in an amount sufficient to dilute the pie 5-fold, i.e., the volume of peptone water equals 4 times the weight of the pie material. Thus, if the pie filling weighed 125 g., a total of 500 ml. of peptone water was added. The latter rather than tap or distilled water was employed as the diluent in this and all subsequent dilutions to prevent destruction of the bacteria *(10)*. The mixture of pie and peptone water is blended for 3 minutes and then used in the reduction test as described below.

Culture medium. Trypticase soy broth (BBL) is distributed in 5 ml. quantities into 15 x 150 mm. screw cap test tubes and sterilized by autoclaving for 10 to 15 minutes at 121° C. Longer periods of autoclaving may decompose the medium with the formation of substances which reduce resazurin independently of bacterial action and interfere with the test. The sterile tubes of media may be stored in the refrigerator for at least a week.

Resazurin solution. This was prepared according to the A.P.H.A. *Standard Methods (1)* by dissolving a standard resazurin tablet in 200 ml. of hot sterile distilled water and cooling. The solution can be stored in the refrigerator for a week without deterioration.

Procedure. Five ml. of the 1:5 dilution of the blended and fully suspended pie material is pipetted into a tube containing the 5 ml. of trypticase soy broth. One-half ml. of the resazurin solution is added and the contents of the tube are mixed by slowly inverting the tube once or twice. Only mild agitation should be used in order to limit incorporation of air which delays reduction of the resazurin. The tube is then placed in a 37° C. water bath so that the contents are completely immersed. Since resazurin is sensitive to light, the water bath is covered with a black cloth. Observations are made every half hour. The contents of the tube are initially blue but turn pink when considerable bacterial growth and reduction of resazurin has taken place. At this point more frequent observations are made, every 15 minutes, until the pink color disappears and the lower ⅓ of the tube is colorless. This is the end point of the test and the time required to reach it is recorded. For convenience, each tube, just prior to immersion in the water bath, is marked with a wax pencil line at one-half inch below the surface of the tube contents. When decolorization of the resazurin reaches this line, the test is considered to have been completed.

Plate counts. The 1:5 dilution of the pie material was diluted further in peptone water in order to make the plate counts used in correlating resazurin reduction times with the actual numbers of bacteria present in the pies. To minimize sampling variations, 50 ml. of the 1:5 dilution of pie was diluted with 50 ml. of peptone water to provide a 1:10 dilution. Subsequent 10-fold dilutions were made by mixing 10 ml. of the previous dilution with 90 ml. of peptone water. Plating was carried out with trypticase soy agar. The plates were incubated at 30° C. for 2 to 3 days and then counted.

CORRELATION BETWEEN RESAZURIN REDUCTION TIMES AND PLATE COUNTS

A total of 77 frozen poultry and beef pies were analyzed for numbers of bacteria and their metabolic reducing activities by means of plate counts and the standard resazurin reduction test. Both determinations were made at the same time on each pie. Representative results are presented in Table 1. It is evident from even a cursory inspection of the data that there is a correlation between reduction times and bacterial numbers. The time required for reduction of resazurin decreases progressively with increase in the bacterial population of the pies. Those with a few thousand bacteria per gram completely reduce resazurin only after an incubation period of 6 to 7 hours whereas pies with several million bacteria per gram require only about 2 hours and intermediate populations give intermediate reduction times.

Closer inspection of the data in Table 1 reveals that the relation between reduction time and bacterial numbers is not always constant. For example, pies 7 and 8 with counts of 99,000 and 29,000 bacteria per gram, respectively, reduced resazurin in the same period of time, 5¼ hours. Likewise, pies 11

TABLE 1

Relation between resazurin reduction times and bacterial plate counts for poultry and beef pies

Pie	Type	Reduction time in hours	Bacteria per gram by plate count
1	Beef	>7¾	1,100
2	Turkey	7	2,600
3	Turkey	6¾	3,900
4	Beef	6¼	12,600
5	Turkey	6	52,000
6	Chicken	5½	62,000
7	Chicken	5¼	99,000
8	Chicken	5¼	29,000
9	Chicken	4½	342,000
10	Chicken	4	211,000
11	Chicken	4	450,000
12	Chicken	4	158,000
13	Chicken	3¾	400,000
14	Chicken	3¾	223,000
15	Chicken	3½	500,000
16	Chicken	3¼	690,000
17	Chicken	2½	3,700,000
18	Turkey	2½	4,100,000[1]
19	Chicken	2½	6,100,000
20	Turkey	2¼	3,700,000[1]
21	Turkey	2	8,800,000[1]
22	Chicken	1	35,000,000[1]

[1] These pies were defrosted and stored at 30° C. for several hours to obtain the high counts.

and 12 with 450,000 and 158,000 bacteria per gram, respectively, gave the same reduction time of 4 hours. This degree of variation is not surprising in view of the basic nature of reduction tests. Different bacterial species and strains are known to vary considerably in reducing ability and variation occurs even with the same strain at different phases of the growth cycle *(1)*. Since precooked frozen pies are exposed to many different sources of bacterial contamination and processing conditions during manufacture, it can be expected that individual pies will vary in their content and proportions of microbial types and in the state of metabolic activity of the bacteria.

These variations do not destroy the usefulness of the reduction test if the latter's limitations in sensitivity are recognized and only fairly broad categories of correlation between reduction times and bacterial numbers are established. Examination of the data on the 77 pies indicated that 3 broad categories or classes could be established in each of which there is a high degree of agreement between reduction times and numbers of bacteria. These classes are as follows:

Class	*Reduction time*	*Bacteria per gram*
1	5 hours or longer	Less than 100,000
2	3 to 5 hours	100,000 to 1,000,000
3	Less than 3 hours	More than 1,000,000

On this basis, the bacterial content of the pies could be predicted from the reduction times with an accuracy of 90 to 95% (Table 2). For example, all of the 22 pies listed in Table 1 can be fitted into these 3 classes without any exceptions. An occasional sample, however, for unknown reasons, reduced the resazurin much faster or slower than would have been expected from the bacterial plate count. More troublesome, however, were a few border-line cases which missed the proper class by 15 minutes.

TABLE 2

Accuracy of the reductase test in the estimation of bacterial counts

Class	Total number of samples[1]	Number of samples correctly classified by the reductase test	Percent accuracy
1	36	33	92
2	21	20	95
3	20[2]	18	90

[1] Based on plate counts.
[2] Most of these pies were defrosted and stored at 30° C. for several hours to obtain the high counts.

The proposed resazurin reduction test should be useful, especially in plant control operations, and public health surveys, even though each of the categories encompasses a fairly large range of bacterial concentrations. There is a trend towards an allowable upper limit of 100,000 viable bacteria per gram of precooked frozen food *(2, 7)*. The rapid test could be used, therefore, to determine whether this suggested standard was being met and also whether excessive contamination was occurring in production. Moreover, as is evident from the data in Table 1, it is frequently possible to obtain closer estimates of bacterial numbers from reduction times than is indicated by the 3 classes. Allowing for variability as described above, the length of the reduction time within each class can be used as a finer measure of the bacterial content. For example, pies that take more than 6 hours for reduction invariably have less than about 10,000 bacteria per gram and those that reduce appreciably faster than three hours have considerably more than 1,000,000 bacteria per gram.

EFFECT OF VARIOUS FACTORS ON THE REDUCTASE TEST

The standard procedure was adopted only after considerable experimentation with a variety of conditions. Results are summarized below in order to indicate the influence of each step in the assay procedure and the permissible variations.

Culture medium. In virtually all reductase tests, except that of Novak *et al. (5)*, which have been applied to milk and other food products, growth of the bacteria has depended upon the nutrients supplied by the food itself. This has certain disadvantages. The food may not contain the type or concentration of nutrients necessary for rapid bacterial growth. Moreover, the composition of even one particular type of food product may vary and thus affect reduction times. The latter situation may be especially important in the case of poultry and meat pies which are heterogenous and variable mixtures of several foods. The importance of adding a culture medium which will support rapid and extensive growth of bacteria to the food sample under assay is evident from the data in Table 3. The addition of trypticase soy broth to the

pie material decreased the time required for resazurin reduction by 2 to 3 hours or approximately 30 to 50%.

Extensive studies of different culture media were not made primarily because past experience had indicated that trypticase soy broth supports good growth of many bacteria. Other complex nitrogenous media may be equally as good or perhaps superior. The trypticase soy medium, however, was found to be better than the acid promoting medium of Novak *et al (5)* which contains peptones, meat and yeast extract and three carbohydrates, and also their skim milk medium. Reduction times with trypticase soy broth were 20%

TABLE 3

Effect of trypticase soy broth on the time required for the reduction of resazurin by chicken pies

Pie	Bacteria per gram	Reduction time (hrs.)		Percent difference
		Pie plus water[1]	Pie plus medium[1]	
1	400,000	5½	3¾	32
2	540,000	6¾	3½	48
3	342,000	6¾	4½	33

[1] 5 ml. of a 1:5 dilution of poultry pie plus 5 ml. of water or trypticase soy broth.

more rapid than with the latter 2 media. A prime difficulty with the acid promoting medium and also with a rapid test based on acid formation is the possible inhibition of bacterial growth by the metabolic acids as they are formed and accumulate. This possibility is supported by our finding that the addition of a small amount of phosphate buffer to the acid promoting medium shortened the time necessary for the reduction of resazurin. Trypticase soy broth contains only a small amount of dextrose, 0.25%, and is buffered with an equal quantity of dipotassium phosphate. As a result, the contents of the assay tube do not change in pH during the entire period of resazurin reduction and remain at the initial pH of 7.2 to 7.4.

Use of trypticase soy broth made up to twice the normal concentration did not decrease resazurin reduction times over those obtained with the normal concentration. Also, there was a greater tendency with the former to give rise to reducing compounds when autoclaved.

Indicators. A number of indicators of the reducing activities of bacteria were compared to resazurin. The single stage color change with methylene blue was not as easy to follow as the 2-stage change with resazurin. Several tetrazolium compounds were also tested. These included 2,3,5-triphenyl-2H-tetrazolium chloride, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride, neotetrazolium chloride, tetrazolium blue and tetrazolium red. They were prepared as 0.1% solutions in sterile distilled water and used in 0.5 ml. amounts per assay tube. Invariably, reduction with the tetrazolium compounds required several hours longer than with resazurin. The iodophenyl compound was unsatisfactory because it turned from colorless to salmon pink immediately on addition to the assay tube and the only further change on incubation was an intensification of the pink color.

Use of 0.1 ml. or 0.25 ml. of the standard resazurin solution per tube rather than the customary 0.5 ml. did not change the reduction time but made it more difficult to follow the color changes. Also, although in some reductase

methods, the first change of resazurin to pink is used as the end point, we found that complete decolorization is a more easily determined and reproducible end point.

Incubation temperature. In comparative assays conducted at 30° C., 35° C., and 37° C., the speed of resazurin reduction increased with increase in the temperature of incubation. Reduction proceeded 40 to 50% more slowly at 30° C. than at 37° C. (Table 4). This is in accord with our kinetic data

TABLE 4

Resazurin reduction times of precooked frozen chicken pies at 37° C. and 30° C.

Pie No.	Bacteria per gram	Reduction time in hours		Increase in time necessary at 30° C. percent
		37° C.	30° C.	
1	10,800	5¼	8	52
2	158,000	4	5¾	44
3	4,300,000	1½	2¼	50

which showed that bacterial multiplication is more rapid at the higher temperature. The bacterial population in the assay tube does not increase during the first hour of incubation. This is the lag period which normally occurs in culture media. After one hour, multiplication of the bacteria begins and then proceeds rapidly. At four-fifths reduction of the resazurin, there are usually about 10 million bacteria per ml. of assay tube contents. On the basis of these data, 37° C. was adopted for use in the standard reductase test.

It is possible that there may be bacteria in the food samples which cannot multiply at 37° C. This temperature was selected simply because resazurin reduction was so much more rapid than at 30° C. Moreover, we were unable to obtain evidence that any appreciable proportion of the microbial population in the pies cannot develop at 37° C. Replicate plate counts of pies in which the plates were incubated at 25° C., 30° C., and 37° C., gave essentially the same numbers of bacteria per gram of pie (Table 5). Incubation of plates at 30° C. was adopted for routine use.

Other factors. Use of less than 5 ml. of the customary 1 : 5 dilution of pie material in the assay lengthened the reduction time. If the concentration of pie material is doubled by use of 5 ml. of a 1 : 2.5 dilution of pie, the reduction time is shortened somewhat but not sufficiently to make up for the disadvantages of working with very thick food suspensions which are difficult to pipette.

Blending of the food samples for 3 minutes is adequate to provide uniform suspensions which can be readily pipetted. Two, 3, and even as many as 6

TABLE 5

Effect of the temperature at which the plates are incubated on the bacterial count [1]

Pie No.	Bacteria per gram at		
	25° C.	30° C.	37° C.
1	480,000	530,000	580,000
2	1,170,000	1,190,000	1,150,000
3	6,300	7,700	6,200
4	5,100	5,900	6,100

[1] All plates were incubated for 3 days prior to being counted.

replicate aliquots taken from such suspensions consistently gave the same reduction time. The results were not altered by blending for as long as 10 minutes. There is a tendency, however, for the food material to heat up during blending and this may eventually lead to destruction of some of the bacteria if the process is continued for too long.

It is important to include the food particles in the assay not only to provide a uniform sample of the bacterial population but also because the particles *per se* may stimulate the growth of the bacteria. Employing the usual assay technique, it was found that an amount of inoculum of *Micrococcus pyogenes* var. *aureus* which reduced resazurin in 5 hours in the absence of any food particles required only 3¼ hours for reduction when sterilized turkey pie material (in the usual concentration) was present in the tube.

DISCUSSION

The pies used in the present investigations were purchased usually from retail stores and were obtained therefore in the frozen state. It is possible that reduction times may be somewhat different if assays are made on the unfrozen product in plants where the pies are produced. The freezing process may injure some of the bacteria in the pies which may delay their multiplication in the assay tube. It seems unlikely, however, that the extent of such possible injury would be sufficient to invalidate the three broad categories of pies proposed. Also the nutrient culture medium added to the food sample can be expected to stimulate recovery of injured cells. In any event, it would appear to be worthwhile to check reduction times against plate counts whenever the method is applied for the first time.

SUMMARY

A rapid resazurin reduction method has been developed which permits the estimation of low, intermediate and high levels of bacteria in precooked frozen foods (poultry and meat pies) within a few hours. An important feature of the method is the addition of a culture medium to the food sample which stimulates bacterial growth and thus speeds reduction of the resazurin. In the standard method, 5 ml. of a 1 : 5 dilution of the blended food sample and 5 ml. of trypticase soy broth are mixed with resazurin and incubated at 37° C. The time required for the mixture to change from blue to colorless is recorded.

The resazurin reduction time and the number of bacteria present were determined for each of 77 pies. On the basis of these data, three classes which correlate bacterial concentrations and reduction times were established. Class 1 contains pies with less than 100,000 bacteria per gram and these require 5 hours or more to reduce resazurin. In Class 2 are pies with 100,000 to 1,000,000 bacteria per gram and these take 3 to 5 hours to reduce the indicator. Finally, in Class 3 are the pies with more than 1,000,000 bacteria per gram and these reduce resazurin in less than 3 hours. The degree of predictability of these three categories from the reductase test ranges from 90 to 95%.

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THE EFFECT OF HEAT UPON CARBOHYDRATE CONVERSIONS IN SWEETPOTATOES[a]

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(Manuscript received April 6, 1957)

Starch in sweetpotatoes is converted through enzymatic hydrolysis to sugars at a rate directly proportional to the temperature of the roots. This hydrolysis has been demonstrated in the studies of Miyaki *(5)*, Culpepper and Magoon *(1)*, and Gore *(2)*. These investigators have shown that the principal increase in sugars resulting from starch hydrolysis is maltose and dextrin.

From previous studies *(3, 4)* the conversion rate of starch to sugars was found to be very rapid between 20° and 65° C. Upon baking, the higher temperature was reached during the first hour; the oven temperature ranged between 149° and 182° C. during this time. This paper reports results of a study undertaken to determine more specifically the temperatures responsible for the most rapid conversion rate.

MATERIALS AND METHODS

Porto Rico and Allgold sweetpotatoes were grown in the horticultural plots at State College, Mississippi, in 1956. Roots from these plots were harvested in early November, and cured for 10 days in temperatures of 30° to 32° C. with a relative humidity of about 90%. After curing, the roots were held in storage at 13° to 18° C. for 3 months. Beginning on February 11, 1957, and for 4 consecutive days, 90 roots of each variety were alternately sampled for carbohydrate content. Two varieties and 180 roots were sampled in each replication; two replications were used.

Lots consisting of 10 roots were sampled raw (at approximately 20° C.) and after being heated to 30, 35, 40, 45, 50, 55, 60 and 65° C. The baking time was approximately 10, 12, 15, 20, 25, 30, 35 and 40 minutes, respectively, for each of the internal temperatures. Temperatures were recorded from thermocouples inserted into the center of the sweetpotatoes. One half of each root in a lot was split lengthwise with a mechanical rasp and a 10-g. sample of the macerated material was placed in 80% ethanol. All samples were then boiled for 15 minutes. Insoluble solids were separated from the ethanol by filtration and determined by drying on a tared filter paper at 65° C. for 2 hours and weighing. Reducing sugars and total sugars in the alcoholic solution were determined by the method of Pickett *(6)* and the non-reducing fraction was determined by subtraction. All of the constituents were reported on a moisture-free basis.

RESULTS

There were marked increases in the sugar content of the roots even at relatively low temperatures, and a corresponding decrease in the alcohol insoluble solids (starch). The rate of change of each constituent was not nearly as great after the roots had been heated to 45° C. The reducing sugar content of the roots at various temperatures is shown in Table 1. Rapid changes occurred in the Porto Rico variety between 20° and 50° C.; whereas, the Allgold roots were heated to 30° C. before rapid increases were initiated. When both varieties were heated above 45° C., the increase in reducing sugars became less with each 5° C. increase in temperature.

Non-reducing sugars increased more rapidly and to greater proportions than the reducing sugar fraction. Initially, a higher level of non-reducing sugars was found in the

[a] Journal article, number 656, Mississippi Agricultural Experiment Station, State College, Mississippi.

raw Allgold sweetpotatoes than in Porto Rico, Table 1. Increases in this fraction were linear in Porto Rico between 20° and 45° C. and between 30° and 50° C. in Allgold. Above 50° C. the increases were irregular and less pronounced for each 5° rise in temperature.

Alcohol insoluble solids decreased linearly as the temperatures increased as shown in Table 1. This constituent amounted to 6% in Allgold, 7% in Porto Rico at 20° C., and decreased to about 3% in each variety at 65° C.

TABLE 1

The effect of heat in hydrolyzing alcohol insoluble solids (starch) to sugars during the baking of sweetpotatoes

Internal root temperatures ° C.	Percentage of dry weight for each variety					
	Reducing sugars		Non-reducing sugars		Alcohol insoluble solids	
	Porto Rico %	Allgold %	Porto Rico %	Allgold %	Porto Rico %	Allgold %
20	3.26	0.49	2.29	10.17	72.1	62.8
30	9.19	1.64	10.47	12.56	55.5	53.3
35	11.05	4.73	12.82	16.60	50.4	49.3
40	13.87	7.29	17.98	17.79	44.5	44.9
45	15.30	10.50	21.10	20.15	41.1	34.9
50	15.98	11.03	20.46	23.60	37.9	33.6
55	15.99	12.44	14.51	20.67	38.8	30.4
60	18.56	12.72	24.57	23.99	30.9	30.0
65	19.34	13.51	24.30	26.18	31.1	29.0
LSD @ 5%	4.88	7.15	7.15	4.53	9.7	7.2

DISCUSSION

Data from this study indicate that the conversion of starch to sugars in sweetpotatoes occurs rapidly at relatively low temperatures. This point is confirmed by the studies of Gore *(2)* and of Sistrunk, Miller, and Jones *(7)*. Differences in the rapidity of hydrolysis below 50° C. for Allgold and Porto Rico might be attributed to inherent differences in the enzymatic systems of the 2 varieties.

Increasing the baking temperature from 20° to 50° C. significantly increased the sugars contained in sweetpotatoes. Temperature effects above 50° C. were more variable than those below this temperature. Such variations were likely caused by uneven cooling of the roots before they were sampled for carbohydrates.

Decreases in the alcohol insoluble solids were caused by the conversion of starch to sugars. In Porto Rico, 23%, 15%, and 9% of these solids were converted to sugars with each 10° C. rise in temperature from 20° to 50°. In Allgold, 15%, 14% and 17% of the alcohol insoluble solids were converted under similar temperature conditions. The total amount of alcohol insoluble solids converted by the time the roots reached 50° C. was 47% for Porto Rico and 46% for Allgold.

Processors interested in developing various kinds of sweetpotato products containing a large amount of the natural sugars should be careful to process the tissue in a manner to take full advantage of the starch to sugar conversion taking place at low temperatures. More research is suggested to determine how high the sugar content can become in various sweetpotato varieties when the roots are processed at temperatures below 45° C.

CONCLUSIONS AND SUMMARY

Sugars increased in Porto Rico and Allgold sweetpotatoes as the internal temperature of the roots increased during baking. Reducing sugars increased from less than 3% in raw roots to 13% in Allgold and to 19% in Porto Rico when heated to 65° C. Non-reducing sugars increased in Allgold from 10% to 26% and in Porto Rico from 3% to 24% under the same temperature conditions. Alcohol insoluble solids decreased as the temperature in the roots increased. Allgold contained 62% and Porto Rico 72% alcohol insoluble solids in raw roots at 20° C. and gradually decreased until each variety contained only 30% at 65° C.

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